2

NZIMLS

THE NEW ZEALAND
INSTITUTE OF MEDICAL
LABORATORY SICIENCE (INC)

ISSN 1171-0195 Volume 74 Number 2 August 2020

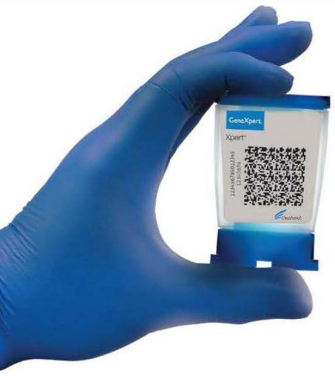
New Zealand Journal of Medical Laboratory Science

Official Publication of the New Zealand Institute of Medical Laboratory Science Incorporated

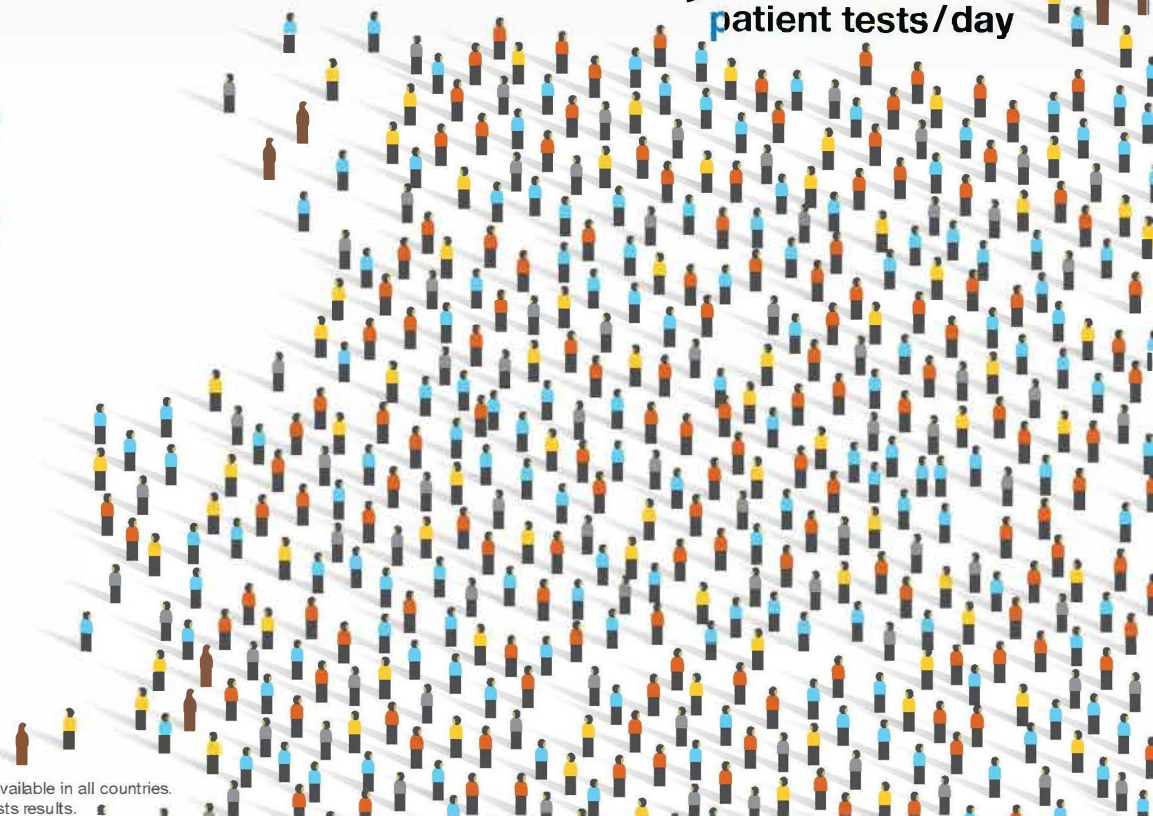
Cepheid
A better way.

Same cartridge, same technology, multiple GeneXpert® Systems from Point of Care to the Laboratory

Ability to scale testing to address patient volume.



48 patient tests/day —— up to ——→ **~2,000** patient tests/day



For more information, contact:

CEPHEID AUSTRALIA/NEW ZEALAND
AUSTRALIA PHONE **1800.107.884**
NEW ZEALAND PHONE **0800.001.028**
CepheidANZ@cepheid.com

www.CepheidInternational.com

About the Journal
The *New Zealand Journal of Medical Laboratory Science* (the Journal) is the official publication of the New Zealand Institute of Medical Laboratory Science (NZIMLS). The Journal is peer reviewed and publishes original and review articles, case studies, technical communications, and letters to the Editor on all subjects pertaining to the practice of medical laboratory science. The Journal is open access (www.nzimls.org.nz/nzimls-journal) and is published three times per year in April, August, and November. Hard copies are circulated to all NZIMLS members and universities and research units in New Zealand and overseas. Current circulation is about 2,200 copies per issue. Printing is by Griffin Press, Christchurch on environmentally responsible paper using elemental chlorine free third party certified pulp sourced from well managed and legally harvested forests and manufactured under the strict ISO14001 Environmental Management System. The Journal is indexed by CINAHL, EMBASE, SCOPUS, Informit, Thomson Gale, EBSCO and Biosis Citation Index, and the Journal Editors are members of the World Association of Medical Editors (www.wame.org).

Brief instructions to authors
The Journal accepts original submissions from anyone and anywhere. Comprehensive instructions can be found on the NZIMLS website (www.nzimls.org.nz/instructions-to-authors.html). All submissions will undergo single-blind peer review and possibly plagiarism checking with iThenticate™ software. If accepted for publication, copyright is vested in the author(s) under terms of the Creative Commons Attribution License (www.creativecommons.org/licenses/by/2.5/legalcode). The authors are responsible for the scientific content and views. Opinions expressed in the Journal are not necessarily those of the Editors, Editorial Board, or Council of the NZIMLS.

Advertising and subscription
Advertisement bookings and enquiries should be addressed to the NZIMLS Executive Officer at fran@nzimls.org.nz. Enquiries regarding subscription and address changes should be addressed to sharon@nzimls.org.nz. Address for the NZIMLS is PO Box 505, Rangiora 7440, New Zealand. Phone +64 3 313 4761.

New Zealand Journal of
Medical Laboratory Science

Volume 74 Number 2
August 2020
ISSN 1171-0195

Editorial
COVID-19 and the New Zealand Institute of Medical Laboratory Science - a view from the bottom up
Terry Taylor 69

Viewpoint
Ethics in medical laboratory practice
Michael Legge 70-71

Original articles
Comparison of MAST CARBA PAcE and an in-house Carba NP for the detection of carbapenemase producing organisms
Julie A Creighton 73-75

Regulatory T cells in patients with lupus nephritis: relationship with disease activity
Douaa Sayed, Mona HA El-Zhori, Halas M Iman, Elbadry I Abo-Elnour, Hossam Elashmawy and Eman NasrEldin 76-81

Lipid ratios are not good predictors of insulin resistance among healthy adults from Southwest Nigeria
Esther Ngozi Adejumo, Adedeji Olusola Adejumo and Omobolanle Abioye Ogundahuns 83-86

Glycaemic control and associated platelet indices among apparently healthy caregivers in Southern Nigeria
Euphoria C Akwiwu, Margaret S Edem, Josephine O Akpotuzor, Idongesit K Isong, Anthony O Okafor and Zibril A Okhormhe 87-90

Crisis frequency and associated changes in platelet parameters among steady state sickle cell subjects
Euphoria C Akwiwu, Eme E Onukak, Idongesit K Isong, Josephine O Akpotuzor, Iya Eze Bassey and Anthony O Okafo 91-94

The diagnostic value of neuron-specific enolase in patients with mild head injury requiring cranial CT scan
Javad Mozafari, Hassan Barzegari, Hassan Motamed, Mohammad Ghasem Hanafi and Ramin Faghihi 95-97

Effect of hypothyroidism on haematological parameters: a gender-based comparison
Samia Karkoutly, Taghrid Hammoud and Faizeh Al-Quobai 98-102

The process control, design and planning stage of ISO15189:2012 management system standard: an implementation update
Dennis Mok and Sharfuddin Chowdhury 103-106

Apoptosis, dendritic cells and their ratio in the peripheral blood of patients with systemic lupus erythematosis
Douaa Sayed, Sonya M Rashad, Essam A Abbada, Ali Sobhy, Mohamed Allam and Eman NasrEldin 107-114

Evaluating the clinical application of nested PCR in the diagnosis of fungal infections
Nasrin Sheikhi, Zahra Rafat, Saeed Zaker Boostan Abad, Sina Mirza Ahmadi, Reza Azizian and Masoud Hamidi 115-118

A New Zealand based quality assurance program for the indirect immunofluorescence antinuclear antibody assay on HEp-2 / HEp-2000 cells. The first year.
Andrew W Soepnel..... 119-125

Gender trends in authorship of articles in the New Zealand Journal of Medical Laboratory Science: 1995-2019
Rob Siebers 126-128

Differences in micronutrient levels between urban and rural children in Cross River State, Nigeria
Nkechi Udo, Iva E Bassey, Uwem O Akpan, Idongesit KP Isong, Faith A Effa, and Augusta Ndudi 129-134

Diagnostic accuracy of the serum biomarker S100A8/A9 and Alvarado score in the diagnosis of appendicitis
Hassan Motamed, Meysam Moezzi, Mohamad Ali Fahimi, Samaneh Porozan, Nastaran Ranjbari and Mohammad Ali Ghiass 135-139

Case studies

Production and disappearance of multiple red cell alloantibodies in a patient with pure red cell aplasia: a case study
Bhavisha Solanki, Raewyn Cameron, Diane Matheson, William Perry and Holly Perry..... 140-143

Identification of *Legionella worsleiensis* in a five-year old male: a case report
Delphine Marjoshi and Aaron Keene..... 144-147

Other features

Abstracts 4th year Otago BMLSc students research projects, Semester 2 148-153

Advertisers 67

English to Māori Clinical Terms 158

Expression of interest: Journal Editor 128

In this issue 67

Journal questionnaires 157

Answers to April Journal questionnaire 147

Journal reviewers August 2019-July 2020 102

NZIMLS Calendar 156

Pacific Way 155

Publications by NZIMLS members 156

Science Digest 154

Advertisers In this issue

Cepheid ..... Inside Front Cover
Bio-Strategy 72
TaG Diagnostics 82
Mediscope 90
Sysmex ..... Outside Back Cover

In this issue

Rob Siebers, Editor

Late February this year the New Zealand medical laboratory workforce was turned upside down due to the Covid-19 pandemic. In a very short time this workforce had to refocus their priorities to deal with Covid-19 testing. In an Editorial, Terry Taylor, President of the New Zealand Institute of Medical Laboratory Science (NZIMLS), informs that the NZIMLS sent a position paper to the Director General of Health with recommendations outlining how medical laboratory scientists can assist with testing assessment and ways to ensure the future stability of both the health response and the protection of frontline diagnostic laboratory services.

There is very little consideration of ethics in medical laboratory science. In a Viewpoint Article, Michael Legge considers the ethical concepts and principles of three analytical phases in the medical laboratory, namely the pre-analytical phase, the analytical phase and the post-analytical phase.

Diagnostic laboratories need rapid and reliable methods to identify carbapenemases and simple tools to differentiate mechanisms of carbapenem resistance. Julie Creighton compared the performance of MAST CARBA PAcE with Carba NP and found that both tests achieved excellent overall sensitivity and specificity.

An imbalance in T helper type 1 response is a major factor in systemic lupus erythromatosis (SLE) pathogenesis. Douaa Sayed and colleagues from Egypt determined the number of $CD4^+$ $CD25^{+/high}$ T cells (Tregs), as well as Foxp3 expression, in peripheral blood mononuclear cells of SLE patients with lupus nephritis and their relations with other activity markers and disease severity. They found an increase in Tregs in patients with active disease which confirms the immunosuppressive nature of these cells and their crucial role in maintaining the immune homeostasis against self-tissues.

Esther Adejumo and colleagues from Nigeria assessed the predictive ability of triglyceride and lipid ratios in predicting insulin resistance among healthy adults. They found that lipid ratios and triglycerides were not useful in predicting insulin resistance.

Akwiwu and colleagues from Nigeria determined glycaemic control of apparently healthy adults whose fasting plasma glucose levels were within the reference range and thus considered normal. Although, no subject had poor glycaemic control, a 9% prevalence was observed for fair glycaemic control and suggestive of pre-diabetes. In this group fasting blood sugar correlated positively with the mean platelet volume, while glycated haemoglobin had a negative relationship with the platelet count. These findings suggests that challenged glycaemic control associates with complications of diabetes with regards to platelet indices even before the onset of diabetes.

A significant aspect of the pathogenesis of sickle cell disease involves inflammation, accompanied by heterocellular leukocyte-platelet-erythrocyte-endothelial adhesive events that

trigger vaso-occlusive episodes, acute organ ischemia, and reperfusion injury. It is therefore plausible to hypothesize on a link between crisis frequency and morbidity indicators, such as a triggered coagulation response. Akwiwu and colleagues from Nigeria investigated crisis frequency of sickle cell anaemia subjects in relation to their platelet parameters and found that an initial increase in crisis frequency brought about depletion in the platelet count and plateletcrit. The authors conclude that an increase in crisis frequency and longer years of living with sickle cell anaemia resulted in higher platelet involvement.

Mozafari and colleagues from Iran investigated the relationship between changes in serum neuron-specific enolase level and changes in CT scan findings in patients with mild head injury associated with intracranial subdural haematoma. They found that serum neuron-specific enolase has a high predictive power to diagnose patients with cerebral hematoma expansion.

Although there are several studies regarding the association between anaemia and hypothyroidism, information is scant about possible correlations between thyroid hormones and haematological parameters. Karkoutly and colleagues from Syria showed that hypothyroidism has a direct effect on most haematological parameters and these effects were more prominent in female patients. In addition, a significant correlation between TSH and RDW was demonstrated in the hypothyroid group. The author suggest that hypothyroid patients, especially women, should periodically have haematological parameters investigated for possible changes.

Mok and Chowdhury provide an update on selected internationally‑oriented guidance documents and relevant literature at the application level that are associated with the implementation of the 'process control, design and planning' stage of ISO 15189:2012. This study contributes to the medical laboratory's development of implementations of ISO 15189:2012 in areas of operations by fulfilling management system and technical competence requirements to an acceptable level of conformance that are regulated by managerial and technical specifications.

Sayed and colleagues from Egypt determined the populations of plasmacytoid and myeloid dentritic cells and to assess apoptosis and the apoptotic cell: peripheral blood dendritic cell ratio of systemic lupus erythematosis (SLE) patients. They found that Inactivated and activated myeloid dendritic cell percentages were decreased in SLE which might be the reason for incomplete removal of apoptotic cells and increase in the early apoptosis/whole myeloid dendritic cell ratio. This clarifies the expansion of apoptosis percentage in SLE patients and might be a vital factor in SLE pathogenesis.

Detection of fungal pathogens is particularly challenging. Sheikhi and colleagues from Iran evaluated the incidence of fungal infections in patients suspected of fungal disease and determined the clinical application of nested PCR for the diagnosis of fungal infections. Their results showed that the incidence of fungal infections was relatively high and dermatophytes were the most common cause of fungal infections. Nested PCR had a high sensitivity and may be useful as a reliable screening method.

Andrew Soepnel from Hamilton reports on the first year of a New Zealand quality assurance program for the indirect immunofluorescence antinuclear antibody assay on HEp-2 / HEp-2000 cells. Excellent inter-laboratory agreement was seen for monospecific and simple mixed patterns. However, some significant patterns, which were supported by second-round specific antibody testing, were missed by some laboratories when present as part of a complex or mixed pattern. He demonstrated how photo submission and a hardcopy specimen free module can provide useful insights.

In a brief communication the Editor, Rob Siebers, In a retrospective analysis of peer-reviewed articles of the New Zealand Journal of Medical Laboratory Science from 1995 to 2019 he found that female authors have been under-represented in publications in the past, but this has increased, especially New Zealand females as first or senior authors.

Udo and colleagues from Nigeria assessed levels of serum vitamin A, iodine, zinc, iron, selenium, total protein, albumin and globulins in urban and rural children in Nigerias. They found that urban children had higher levels of macronutrients and lower levels of micronutrients than rural children related to a high prevalence of malnutrition.

Appendicitis is one of the most common causes of patients referring to emergency departments with abdominal pain. Motamed and colleagues from Iran investigated the diagnostic accuracy of the serum biomarker S100A8/A9 in diagnosing appendicitis. They found that serum levels of S100A8/A9 have a low sensitivity and specificity; therefore, they have no diagnostic value in appendicitis.

Alloimmunisation against red cell antigens poses a challenge to blood transfusion. Solanki and colleagues present a case study of a patient who was transfusion-dependent due to pure red cell aplasia, produced multiple alloantibodies against antigens of three blood group systems. The persistence of the alloantibodies in this patient was much shorter than expected.

Delphine Marjoshi and Aaron Keene report a case of *L. worsleiensis* infection causing Legionnaires' disease in a five-year old male. The clinical presentation resembled infections caused by other species of *Legionella*. The diagnosis was made using PCR and the bacterial species was identified by 16S rRNA sequencing. Two other family members also presented with similar symptoms, however, the presence of *L. worsleiensis* could not be established. This is believed to be the first case of *L. worsleiensis* infection in a child in New Zealand. Furthermore, this case may be part of a point-source outbreak, which has never been reported for this species.

Correction

Adriaansen M, Williams S, van den Boom J. Evaluation of two transcutaneous bilirubin devices for the assessment of neonatal jaundice in a diverse New Zealand population. N Z J Med Lab Sci 2020; 74: 11-16.

Unfortunately, in the print copy, Figure 3 was supposed to be the Bland-Altman plot of Bilicare vs total bilirubin but instead Figure 2 was duplicated here. It has been corrected in the online version.

EDITORIAL

COVID-19 and the New Zealand Institute of Medical Laboratory Science - a view from the bottom up

Terry Taylor

Who knew when we were all celebrating the starting of a new decade what was going to unfold within three months early in 2020. We watched as pictures and dialogue from Wuhan, China showed a health system under strain from an acute respiratory illness. Within 6 weeks the virus we call COVID-19 had spread to all corners of the world carried on all forms of transport. New Zealand's first case was identified on 28th February, by March 12th the World Health Organization had announced a global pandemic, and by the time of the level 4 lockdown on March 26th New Zealand's number of confirmed cases was 283 and rising exponentially.

Laboratory workers all over New Zealand had already started preparation for the impending lockdown. Shifts were changed and we started to work in pods to ensure that if any of the workers got infected or were contacts the laboratory could still maintain testing and general cover. There was an urgent push to setup and validate COVID-19 PCR testing in laboratories throughout New Zealand. The work and expertise to get this testing operational in a short space of time was exceptional. Many laboratory workers moved away from their homes to alleviate the associated risk to family and community. On Friday, March 27th the New Zealand Prime Minister, the Rt Hon Jacinda Ardern, gave a personal thankyou to the medical laboratory workers for stepping up in the frontline of the health response to COVID-19. Suddenly this quiet group of dedicated health professionals was thrust into the limelight.

By May 28th our New Zealand scientists and technicians have processed over 267 000 COVID-19 PCR tests with a testing rate of 5.6% (Ministry of Health data). The nationwide capacity for COVID PCR testing is around 10000 tests per day (Ministry of Health data). This accurate and efficient work has been critical in providing reliable modelling data and re-assurance for contact tracing. This has assisted in the decision for New Zealand to move from level 4 to level 3 on the 23rd April and will be used as we move towards alert level 1 and normality.

Since March 2020 the New Zealand Institute of Medical Laboratory Science (NZIMLS) has had enquires from most of the major news outlets about all aspects of COVID-19 testing and what exactly the medical laboratory science profession is all about. Some of our members have also been interviewed for a documentary on the New Zealand COVID-19 laboratory response. There has been regular dialogue with New Zealand politicians, health experts and agencies about our professional role in the epidemic response. The NZIMLS put in a submission to the Epidemic Response Committee, which was set up as a select committee to hold the government to account while parliament was unable to sit during lockdown. COVID-19 has thrust the profession of medical laboratory science as an essential frontline health service in both the public and political eye.

As New Zealand moved into alert level 2 during mid-May, the NZIMLS sent in a position paper to the Director General of Health, Dr. Ashley Bloomfield and all the relevant government ministers reiterating the outstanding work our medical laboratory workers were providing in challenging circumstances. There were recommendations for utilising medical laboratory scientist expertise within governance at the Public Health, District Health Boards and Ministry of Health level. The recommendations outlined how scientists can assist with testing assessment and ways to ensure the future stability of both the health response, but also the protection of frontline diagnostic laboratory services.

Medical laboratory scientists are, as a rule, quiet back room achievers which unfortunately in the past has not helped promote our public and political profile, nor gave us opportunities for direct governance representation at the District Health Board or Ministry of Health level. As a result, medical laboratory workers in New Zealand have in the past 15 years endured some of the most difficult times with our past concerns and advice constantly overlooked in contract and service restructures. There is an expectation that NZIMLS members will have an involvement with post COVID-19 debriefs and future workforce planning as a direct result of this recent dialogue.

Now is clearly the time to take advantage of the increase in the profile of medical laboratory workers. All practitioners need to take every opportunity to remind decision makers and influencers that laboratory testing is essential and a critical factor in providing quality healthcare decisions that benefit all New Zealanders.

Kia Kaha Aotearoa

AUTHOR INFORMATION

Terry Taylor, BSc DipMLS, Medical Laboratory Scientist[1] and President[2]

[1]Southern Community Laboratories, Dunedin
[2]NZIMLS, Rangiora

Correspondence: Terry Taylor
Email: president@nzimls.org.nz

VIEWPOINT

Ethics in medical laboratory practice

Michael Legge

INTRODUCTION

Despite a considerable literature relating to "ethical practice", there is very little consideration of ethics in medical laboratory science. In general, it is accepted that there are guiding principles and these are reflected in the NZIMLS "Code of Ethics". Where do these broad guiding principles come from and how might they relate to everyday ethical practice? The word bioethics is derived from the Greek words *bios* (life) and *ethike* (ethics). However, it was not the Greeks who created bioethics in the modern context, nor was bioethics concerned as it is now. In 1920 a German pastor (Fritz Jahr) defined "bio-ethike" as a compassionate attitude towards animals and plants based on scientific research. In 1970 the concept was later rediscovered by an American biochemist, Van Renslaer Potter, who defined "Bioethics' as a new science of survival He concludes:" Man's survival may depend on ethics based on biological knowledge, hence Bioethics". Today there are many definitions or concepts applied to ethics, however three simple definitions can be applied across the ethical arena. Ethics: rules of behavior based on ideas of what is morally good and bad. Bioethics: philosophical, social and legal issues arising in medicine and the life sciences. Medical ethics: moral values and judgments as applied to medicine e.g. The Hippocratic Oath taken when qualifying in medicine attributed to Hippocrates (460 to 370BC), although a more modern version is now used. While ethics is viewed as a relatively modern concept in the 19th century "medical ethics" was viewed as a process of limiting disputes and a means of limiting patient's options for alternative treatment, giving rise to the self-governing UK authority the General Medical Council.

Important ethics milestones in the twentieth century

For modern bioethical concepts and principles three important ethics milestones evolved. After the Second World War in 1946, NAZI officials and SS scientists were put on trial for involuntary euthanasia of millions of people and for human experimentation in the concentration camps. Such experiments were conducted without consent and unjustifiable 'scientific' procedures. From these trials the Nuremberg Code (1947) was developed which had the basic principle that consent from any subject to medical and scientific procedures must be voluntary and informed. Subsequently in 1947 the" Declaration of Geneva" produced a Code of Practice for the medical profession. Based on these two important ethical milestones, in 1964 the "Declaration of Helsinki" was written. This document focused on well-being, written consent and the right to independent assessment, and is regularly reviewed (now in the seventh version) and updated to keep pace with changing trends in medicine and sciences. Importantly it is not a law. As a consequence of the defining of ethics, bioethics has emerged as a discipline focusing on biomedical research and clinical practice rather than the early concept of the "natural world". During these early years there was considerable debate surrounding what "ethical principles" were and how they should be applied. In 1985 two Americans (Tom Beauchamp and James Childress) proposed as the basis for medical ethics four moral principles: Autonomy and protection i.e. a moral obligation and respect for persons; Beneficence and Non-maleficence i.e. the best interests of patients and research subjects and Justice i.e. treating patients equally and respect for morally acceptable laws. These four ethical principles are embedded in the NZIMLS "Code of Practice". It is important however, to realize that ethical principles are not law although they can clearly be applied in law.

Common areas of ethical applications

The 'big picture' ethical applications often appear in the media or are promoted by bioethicists as issues with significant ethical outcomes. While it is not intended to discuss these, they would include typical areas such as euthanasia, end of life care, eugenics, organ donation, reproductive technologies, genetic testing and human experimentation. All of these have had both individually and collectively extensive public discussion, debate and a significant number of publications.

Everyday ethics

In everyday practice many aspects of work practice can be considered in ethical concepts. Consent, underpinning the rights to be informed; Confidentiality, the right to be respected; Honesty, to apply truthfulness to any procedure or information; Conflict of interest, to ensure professional judgement is not unduly influenced by a secondary factor e.g. financial gain; Proficiency, to exhibit a high degree of skill and expertise; Medical actionable results, ensuring results are correct and both medical and laboratory errors are avoided or identified; Professionalism, working to the appropriate standards as scientists and technicians; Codes of Conduct, the rules to follow that will encompass all of the above considerations and incorporate these into everyday practice.

The everyday practice extends into other significant laboratory practice such as publishing i.e. ensuring data are correct and plagiarism has not occurred; genetic testing, many laboratories may not have policies on testing and communicating these results or control how the patient is informed. Patients records, unauthorized access to patients records involves issues of privacy and dishonesty if communicating information to a third party. A similar issue relates to accessing and communicating autopsy results.

Everyday ethics in the laboratory

In considering the ethical concepts and principles indicated earlier in this essay, how might these be applied to the three principle analytical phases in the laboratory? From the ethical principles as proposed by Beauchamp and Childness indicated earlier in this essay, three are significant.

Pre-analytical phase

Respect for the person i.e. identification and consent, right to refuse and confidentiality. Beneficence i.e. test should benefit the patients, there is no intentional harm and any samples for research only are with informed consent. Justice i.e. there is equity for all patients.

Analytical phase

Respect for the persons i.e. the right to refuse and confidentiality which would also apply to Point of Care testing. Beneficence i.e. good laboratory practice, proper and appropriate accreditation, refusing to accept test reports if compromised. Justice i.e. ensuring equity for all patients and appropriate test turn-around times.

Post-analytical phase
Respect for persons i.e. confidentiality, patients right to results access and involvement with results, patients right to refuse further testing. Beneficence i.e. results are interpreted by an appropriately qualified person, all errors and notified. Justice i.e. all results are treated equally, there is a policy on the fate of residual samples.

While it is clear there is overlap in these concepts and principles it is important to recognize that the application of bioethics crosses all boundaries in both medicine and pathology.

Violation of ethical principles
There are many cases where ethical principles involving human subjects have been violated. There are however, five which stand-out and have been defining moments in the development of modern bioethical consideration.

The Doctors Trials (1946): These were the infamous Nuremberg Trials of 23 NAZI doctors and scientists for human experimentation and involuntary euthanasia giving rise to the Nuremberg Code indicated earlier in this essay.

Tuskegee Experiment (1932 to1972): 350 African-Americans were deliberately and unknowingly infected with syphilis and remained untreated to study the progress and effects of the disease. Many of those infected passed on syphilis to their wives and children.

Willowbrook School Experiment (1956 to 1970): The school in the USA was for intellectually handicapped children who were deliberatively infected with hepatitis to study the course of the disease and development of a possible immunotherapy. Their parents were not informed.

American Cancer Society Experiment (1970s): Rest home residents were deliberatively injected with cancer cells to study cancer outcomes. Neither the residents or their relatives were informed.

These and other significant ethical concerns in the USA led to the Belmont Report in 1978 which defined ethical principles in health care based on respect for persons, Beneficence and Justice.

The Unfortunate Experiment (1966 to 1982): A New Zealand case where women with cervical cancer were untreated to study the course of the disease without their consent. This subsequently led to the Cartwright Enquiry and resulted in a major transformation of human bioethics in New Zealand.

CONCLUSION

Ethics, Bioethics, Medical Ethics are all founded on the same basic principles but are interpreted according to their respective working concepts. In patient care they all adhere to the modern foundation that created ethical practices. If the laboratory seems some distance from ethical history and theories it should not be forgotten that the best interests of the patient or research subject should be at the core of everyday ethics in the everyday workplace.

In concluding perhaps consider whether the following poses any ethical issues for the laboratory? In a large teaching and research-based hospital two teams have developed what they consider a solution to organ transplants. Team one has grown kidneys from stem cells and Team two has produced “humanised” body organs from genetically engineered pigs. To ensure either are safe for transplants they use clinically dead but “kept alive” persons to test the use of the ‘engineered” body organs. Blood and other samples are sent to the laboratory to verify the success (or otherwise) of he two sets of transplants. Two ethical questions for consideration. If the person(s) are dead they have no rights, therefore are the transplant teams justified in the experiments and second what would the position be of the laboratory in accepting samples and subsequent analysis?

Note: This essay is based on the presentation “Ethics in Medical Laboratory Practice” that would have been made at the South Island Seminar, March 28th which was cancelled due to COVID-19.

AUTHOR INFORMATION

Michael Legge, PhD MRSB FIBMS FNZMLS FFSc(RCPA)
Associate Professor
Department of Biochemistry, University of Otago, Dunedin

Correspondence: michael.legge@otago.ac.nz

Fellowship of the NZIMLS

The NZIMLS encourages members to consider Fellowship as an option for advancing their knowledge and career prospects. Fellowship provides an attractive option to academic postgraduate qualifications at a fraction of the cost.

Fellowship of the NZIMLS may be gained by thesis, by peer reviewed publications; or by treatise in case of a member holding an appropriate postgraduate or professional qualification.

Fellows may use the nominals FNZIMLS if a current financial member of the Institute.

THESIS

The thesis must be based on the style of Master of Science by thesis requirement of New Zealand universities.

PUBLICATIONS

A minimum of ten peer reviewed publications in international or discipline acknowledged biomedical journals. The candidate must be the 1st or senior author of at least six of these publications. A comprehensive review of the submitted publications is also required.

TREATISE

By submission of a treatise in the form of a dissertation of 3000 5000 words on a medical laboratory science subject. The dissertation may take the form of a review, a scientific study, development of a hypothesis, or any other presentation that meets with the approval of the Fellowship Committee. Candidates applying for Fellowship by this route must be holders of at least a Master’s degree or higher in a medical sciences subject, or have a professional qualification such as Fellowship of the following professional bodies: Australian Institute of Medical Science; Institute of Biomedical Science; Faculty of Science of the Royal College of Pathologists of Australasia, Australiasian Association of Clinical Biochemists; Royal Institute of Biology, London.

For full Fellowship regulations and the application process visit the NZIMLS web site at www.nzimls.org.nz

The current fee for sitting Fellowship is $575.00 (incl. GST)

NeuMoDx™ MOLECULAR SYSTEMS

REVOLUTIONARY MOLECULAR DIAGNOSTIC SOLUTION

Intuitive 3-step workflow
Streamlined workflows give unprecedented ease of use, reduced hands-on time and minimized training burden

True random access
Continuous loading of samples and up to 30 IVD assays can be run in parallel—no need to batch

IVD & LDT in parallel
Seamless and LDT* tests in the same workflow

*LDT: laboratory developed test or in house tests

High throughput
The NeuMoDx 288 can process up to 340 samples in an 8-hour shift and the NeuMoDx 96 up to 145 samples

Ultra-fast insights
Results available in less than one hour for most tests with true STAT functionality

No reagent reconstitution
Proprietary NeuDry test strip reagents and consumables are ready to use and room temperature stable, stored or on board

NeuMoDx™ Molecular Systems provide the industry's first true continuous random-access solution and is scalable to meet the needs of the modern clinical laboratory. The NeuMoDx 96 and NeuMoDx 288 Molecular Systems are fully automated analyzers that use proprietary NeuDry™ technology, magnetic particle affinity capture, and real-time PCR chemistry in a multi-sample microfluidic cartridge. This unique integration of robotics with advanced microfluidics delivers industry-leading usability, enhanced performance, increased efficiency, and rapid turnaround.

bio-strategy
delivering technology

for more information please contact
www.bio-strategy.com | T: 0800 34 24 66

ORIGINAL ARTICLE

Comparison of MAST CARBA PAcE and an in-house Carba NP for the detection of carbapenemase producing organisms

Julie A Creighton

ABSTRACT

Objectives: Diagnostic laboratories need rapid and reliable methods to identify carbapenemases and simple tools to differentiate mechanisms of carbapenem resistance. Issues with false negative results and difficulties with detection in non- Enterobacterales mean that the ultimate phenotypic test has yet to be discovered. MAST Group have recently released the CARBA PAcE assay; a rapid 10-minute enzymatic test. This study compares the performance of MAST CARBA PAcE with Carba NP.

Materials and methods: 200 isolates were included in the study: 168 Enterobacterales, 19 *Pseudomonas aeruginosa*, and 13 *Acinetobacter baumannii*. 123 carbapenemase-producing organisms (CPO), consisting of KPC (*n*= 8), IMI (*n* = 2), NDM (*n* = 50), VIM (*n* = 7), IMP (*n* = 7), OXA-48-like (*n* = 34), OXA-23 (*n* = 8), one each of OXA-181/NDM, OXA-48/NDM, VIM/IMP, OXA-24, OXA-25, OXA-27, OXA-58; and 77 non-carbapenemase producing isolates.

Results: Both the Carba NP and PAcE performed with excellent sensitivity and specificity of 99.2%/100% and 97.6%/98.7% respectively. Both tests failed to detect an IMI-2-producing *Enterobacter cloacae*, with the PAcE also failing to detect an NDM-1-producing *Providencia stuartii* and an OXA-48-producing *E. coli*. One hyper K1-producing *Klebsiella oxytoca* was false positive in the PAcE assay. All *P. aeruginosa* and *A. baumannii* carbapenemase producers were detected by both methods.

Conclusions: This study has demonstrated that the PAcE and Carba NP tests achieved excellent overall sensitivity and specificity. Although the number of glucose-nonfermenting isolates were small, both assays successfully detected 100% of carbapenemase producing *P. aeruginosa* and *A. baumannii*. PAcE has a time advantage, but weak positive results can cause interpretation problems and the product shelf life might be a limitation for laboratories that have low numbers of carbapenem resistant organisms. The diversity and expression of carbapenemase enzymes requires laboratories to employ more than one phenotypic method for carbapenemase detection.

Key words: MAST CARBA PAcE, Carba NP, carbapenemase

N Z J Med Lab Sci 2020; 74: 73-75.

INTRODUCTION

According to the Centers for Disease Control and Prevention (CDC), in their 2019 Antibiotic Resistance Threats Report (1), more than 35,000 people in the United States of America (USA) die each year as a consequence of over 2.8 million antibiotic-resistant infections. That equates to a staggering 100 people each day – in the USA alone. Among the most urgent threats are carbapenem-resistant *Acinetobacter baumannii* and carbapenem-resistant Enterobacterales (CRE). Of concern to the CDC is escalating community-acquired infections, which can be challenging to identify and contain any spread (1).

The most concerning carbapenem-resistant organisms are those that harbour carbapenem hydrolysing enzymes: a diverse group of carbapenemases that can be found in many different Gram-negative bacilli, with an escalating prevalence and global dispersion. Routine diagnostic laboratories need rapid and reliable methods of enzyme identification and simple, cost-effective tools to differentiate between organisms that are resistant to carbapenems by means of a carbapenemase or because of ESBL/hyper AmpC production combined with outer membrane impermeability and/or up-regulated efflux pumps. Although there are a variety of phenotypic tests available, they are prone to false negative results due to weakly expressing carbapenemases, such as OXA-48-like and OXA-23, multiple resistance mechanisms and detection in non- Enterobacterales (2,3). The ultimate phenotypic test has yet to be discovered and commercial molecular systems cannot identify novel carbapenemases.

MAST Group (Liverpool, UK) have recently released the CARBA PAcE test (PAcE); a rapid 10-minute test, based on enzyme hydrolyses of an indicator carbapenem and conversion of a pH indicator from yellow to red, and shades thereof. The aim of this study was to compare the performance of MAST CARBA PAcE with that of an in-house Carba NP reagent for detection of carbapenemase production in Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

MATERIALS AND METHODS

Bacterial isolates

A total of 200 unique patient isolates were included in the study: 168 Enterobacterales, 19 *Pseudomonas aeruginosa*, and 13 *Acinetobacter baumannii*. The study isolates included 123 carbapenemase-producing organisms (CPO), consisting of KPC (*n*= 8), IMI (*n* = 2), NDM (*n* = 50), VIM (*n* = 7), IMP (*n* = 7), OXA-48-like (*n* = 34), OXA-23 (*n* = 8), one each of OXA-181/NDM, OXA-48/NDM, VIM/IMP, OXA-24, OXA-25, OXA-27, OXA-58; and 77 non-carbapenemase producing isolates, including 9 wild-type *E. coli* and 68 multi-drug resistant strains; 25/68 (36.8%) of which were non-susceptible to one or more carbapenem. KPC-producing *Klebsiella pneumoniae* BAA1705 and ESBL-producing *K. pneumoniae* BAA1706 were used as positive and negative controls and tested with each batch. Isolates were stored at -70°C and subcultured onto Columbia base Blood Agar, then Mueller-Hinton agar before testing. Any discrepant results were retested using a fresh rapidly-growing culture harvested adjacent to a carbapenem disc.

MAST CARBA PAcE

PAcE was performed according to guidelines supplied by the manufacturer. Briefly, 1-5µl of test organism was emulsified in 250µl of reconstituted test solution, vortexed for 20 seconds, then incubated for 10 minutes at 35°C. Test results were read

after 10-15 minutes incubation. A positive result was indicated by a colour change from yellow to orange/red. Colour images for comparison purposes are provided in the pack insert.

CARBA NP

Carba NP was performed as previously described (4). Briefly, 100µl of Tris-HCL lysis buffer (Thermofisher) was added to two tubes (A and B). Approximately 10µl of test organism was emulsified into each tube. 100µl of a buffered phenol red solution was added to tube B (control tube) and 100µl of imipenem-supplemented solution was added to tube A. Tubes were incubated at 35°C and read at intervals of 10-, 60- and 120-minutes. A positive result was indicated by a colour change from red to yellow/orange in tube A, with tube B remaining red. Any discrepant results were retested and incubated for up to 3 hours. For *A. baumannii* lysis buffer was replaced with saline.

RESULTS

Overall, the Carba NP and PAcE tests performed with excellent sensitivity and specificity: 99.2% / 100% and 97.6% / 98.7% respectively (presented in Table 1). Both tests failed to detect an IMI-2-producing *Enterobacter cloacae*, with the PAcE also failing to detect a NDM-1-producing *Providencia stuartii* and an OXA-48-producing *E. coli*. There were no false positive results with Carba NP, but one hyper K1-producing *Klebsiella oxytoca* was repeatedly strongly positive with PAcE. All *P. aeruginosa* and *A. baumannii* carbapenemase producers were detected by both Carba NP and PAcE, with no false positive results (100% sensitivity and 100% specificity) (Table 2). At test completion (10-15 minutes) the PAcE assay produced 10/120 (8.3%) weak or very weak positive results, whereas the Carba NP had 3/122 (2.4%) weak positive results after 2 hours incubation.

Table 1. Performance of Carba NP and MAST PAcE assays.

		No. positive	
Study isolates	**No. tested**	**Carba NP (%)**	**MAST PAcE (%)**
Carbapenemase			
KPC	8	8 (100%)	8 (100%)
IMI	2	1[a] (50%)	1[a] (50%)
NDM	50	50 (100%)	49[b] (98%)
VIM	7	7 (100%)	7 (100%)
IMP	7	7 (100%)	7 (100%)
OXA-48-like	34	34 (100%)	33[c] (97.1%)
OXA-23	8	8 (100%)	8 (100%)
OXA-other	4	4 (100%)	4 (100%)
Dual	3	3 (100%)	3 (100%)
Non-carbapenemase	77	0 (0%)	1 (1.3%)
Overall sensitivity	123	122 (99.2%)	120 (97.6%)
Overall Specificity	77	0 (100%)	1 (98.7%)

False negative results: [a] IMI-2-producing *Enterobacter cloacae*; [b] NDM-1-producing *Providencia stuartii*; [c] OXA-48-producing *E. coli*.

Table 2. Performance of Carba NP and MAST PAcE by genus.

	No. tested		Carba NP Sensitivity (%)	Specificity (%)	MAST PAcE Sensitivity (%)	Specificity (%)
Study isolates	**CPO**	**Non-CPO**				
Enterobacterales	102	66	99.0	100	97.1	98.5
P. aeruginosa	10	9	100	100	100	100
A. baumannii	11	2	100	100	100	100

DISCUSSION

This study compared the performance of Carba NP and PAcE against a collection of 123 carbapenemase-producing isolates and 77 predominantly multi-drug resistant non-carbapenemase-producing isolates. We have demonstrated that both methods perform extremely well, achieving excellent sensitivity and specificity for the detection of carbapenemase production in Enterobacterales, *P. aeruginosa* and *A. baumannii*. Among 123 CPO test isolates, Carba NP test achieved an overall sensitivity of 99.2%, with a specificity of 100%; and PAcE test achieved an overall sensitivity of 97.6%, with one false positive, resulting in a specificity of 98.7%.

OXA-48-like are one the most commonly found carbapenemases in New Zealand (5). Pitout *et al.* (6) has ominously described these enzymes as a "looming threat", due to their global emergence, spread and difficulties with laboratory detection. OXA-48-like carbapenemases hydrolyse penicillins at a high level, but only weakly hydrolyse carbapenems, with concerns that some strains produce a meropenem MIC below the EUCAST screening cut-off (7). Thus, many phenotypic assays struggle to achieve high detection sensitivity (2, 8). Therefore, it is reassuring that both Carba NP and PAcE detected these enzymes with a high-level of sensitivity [Carba NP 100% (34/34) and PAcE 97.1% (33/34)]. However, it is noteworthy that many of the OXA-48-producing isolates provided weak or very weak colour changes in both tests (data not shown), potentially causing subjective results and interpretation problems. Colour development with PAcE became stronger after an extra 10-15 minutes at room temperature, and weak Carba NP positive results became stronger after extended incubation to 3 hours, with no additional false positive results. The manufacturer's instructions for PAcE does allow for reading up to 20 minutes after incubation.

Our study included 50 NDM producing isolates (48 Enterobacterales and 2 *P. aeruginosa*), all of which were detected by Carba NP, with the PAcE test failing to detect one NDM-1-producing *Providenica stuartii*. Other authors have reported problems with the detection of NDM enzymes in the Morganellaceae family (8-10), with issues likely due to low enzyme expression or inadequate zinc in the growth medium used for investigations.

Detection of carbapenemase in non-glucose-fermenting organisms is a widely accepted challenge (3,11). *Pseudomonas spp*, particularly in a hospital setting, are often multi-drug resistant, with carbapenem resistance more often caused by non-enzymatic mechanisms such as outer membrane permeability changes and efflux pump upregulation, rather than a carbapenemase. On the other hand, carbapenem and multi-drug resistant *Acinetobacter spp* are more likely to be harbouring an oxacillinase such as OXA-23, which can be problematic to detect due to weak carbapenemase hydrolysis, lack of a specific indicator agent and non-inclusion in some commercial molecular tests (3,11). Although our study included only small numbers of glucose-nonfermenting isolates, it is encouraging that both Carba NP and PAcE successfully detected 100% of carbapenemase producing *P. aeruginosa* and A. baumannii, with no false positive results.

No false positive results were found with the Carba NP, which concurs with various other investigations (2,3,12). In contrast the PAcE test repeatedly yielded a strong false positive result with a hyper K1-producing *K. oxytoca*. It could be argued that this isolate was susceptible to carbapenems and would not otherwise be tested for the presence of a carbapenemase; however, it is important to identify potential issues with false positive reactions as it is possible for organisms to coproduce different β-lactamase enzymes. Interestingly *K. oxytoca* has been found to cause false positive reactions in other colourimetric assays. Meier *et al.* (13) observed three *K. oxytoca* isolates, harbouring different β-lactamases, that produced false-positive results in three colourimeteric tests (β-Carba, bcCarba NP, and NeoRapid CARB). The authors suggested the false-positive reactions could be attributed to the procedure involved with testing directly from blood culture broths or linked to the species itself (13). However, Noël *et al.* also reported a strain of *K. oxytoca* that was responsible for a false-positive with the β-Carba test, when tested from solid media (11).

The limitations of this study include the low number of non-glucose-fermenting organisms evaluated and the limited diversity of carbapenemase enzymes tested. However, the predominance of OXA-48-like and NDM carbapenemases reflects the current epidemiology in NZ (5). The Carba NP method has been used at our institution for nearly 5 years therefore familiarity with the colour change interpretation to indicate a positive result could contribute to this test being less subjective than for other users who have less experience.

To the best of our knowledge this is the first evaluation of the MAST CARBA PAcE test. Our evaluation found a high level of performance in terms of sensitivity and specificity for the detection of carbapenemase production in Enterobacterales, *P. aeruginosa* and *A. baumannii*. The PAcE test is simple to perform with advantages over Carba NP including rapid time to results (10 – 15 mins compared to 2 hours) and the kit is commercially prepared. Disadvantages for both assays include the subjective colour change for weak positive results, especially for isolates harbouring OXA-48-like enzymes, and an inability to differentiate carbapenemase type.

For PAcE an additional disadvantage is the short shelf-life of the reconstituted test solution, which must be used within 30 days. Preliminary testing of stored reagent at CHL over the last 6 months has shown promising results for -70^{o}C long term storage. Further studies could be conducted to determine if reconstituted PAcE reagent could be aliquoted and frozen at -70^{o}C to prolong the shelf life, thus making the product more cost effective for laboratories who rarely experience carbapenem resistant isolates. The diversity and expression of carbapenemase enzymes requires laboratories to employ more than one phenotypic method for carbapenemase detection.

ACKNOWLEDGMENTS

Grateful thanks to Kerry Latham, Scientist, CHL, for assisting with the laboratory testing of all test isolates. Fort Richard Laboratories, Auckland, supplied some of the reagents and consumables used for this study. Part of this evaluation was presented as a poster at the 2020 Australian Society for Antimicrobials meeting, Melbourne, Australia.

AUTHOR INFORMATION

Julie A Creighton, DipMLT FNZIMLS, Senior Medical Laboratory Scientist[1] and Clinical Lecturer[2]

[1]Department of Microbiology, Canterbury Health Laboratories, Christchurch, New Zealand
[2]University of Otago, Christchurch, New Zealand.

Correspondence: Julie Creighton, Microbiology Laboratory, Canterbury Health Laboratories, P. O. Box 151, Christchurch, New Zealand.
Email: julie.creighton@cdhb.health.nz

REFERENCES

1. www.cdc.gov/drugresistance/biggest-threats.html (Accessed 18 November 2019).
2. Tamma P, Simner P. Phenotypic detection of carbapenemase-producing organisms from clinical isolates. *J Clin Microbiol* 2018; 56: e01140-18.
3. Simner P, Opene B, Chambers K, Naumann ME, Carroll KC, Tamma PD. Carbapenemase detection among carbapenem-resistant glucose-nonfermenting Gram-negative bacilli. *J Clin Microbiol* 2017; 55: 2858-2864.
4. Creighton J, Jayawardena S. Comparison of four phenotypic tests, three biochemical tests and Cepheid Xpert Carba-R for detection of carbapenemase enzymes in Gram-negative bacteria. *N Z J Med Lab Sci* 2015; 69: 81-85.
5. Institute of Environmental Science and Research Ltd. *Enterobacterales* with acquired carbapenemases, 2018. Porirua, New Zealand: ESR; 2018. Available at https://surv.esr.cri.nz/PDF_surveillance/Antimicrobial/ACE/2018Carbap.pdf
6. Pitout JD, Peirano G, Kock MM, Strydom KA, Matsumura Y. The global ascendency of OXA-48-type carbapenemases. *Clin Microbiol Rev* 2019; 33: e00102-e00119.
7. Hopkins KL, Meunier D, Mustafa N, Pike R, Woodford N. Evaluation of temocillin and meropenem MICs as diagnostic markers for OXA-48-like carbapenemases. *J Antimicrob Chemother* 2019; 74: 3641-3643.
8. Osei Sekyere J, Govinden U, Essack SY. Review of established and innovative detection methods for carbapenemase-producing Gram-negative bacteria. *J Appl Microbiol* 2015; 119: 1219-1233.
9. Saleh A, Göttig S, Hamprecht AG. Multiplex immunochromatographic detection of OXA-48, KPC, and NDM carbapenemases: impact of inoculum, antibiotics, and agar. *J Clin Microbiol* 2018; 56: e00050-18.
10. Baeza LL, Pfennigwerth N, Greissl C, Göttig S, Saleh A, Stelzer Y, et al. Comparison of five methods for detection of carbapenemases in Enterobacterales with proposal of a new algorithm. *Clin Microbiol Infect* 2019; 25: 1286-e9.
11. Noël A, Huang TD, Berhin C, Hoebeke M, Bouchahrouf W, Junus S, et al. Comparative evaluation of four phenotypic tests for detection of carbapenemase-pro ducing Gram-negative bacteria. *J Clin Microbiol* 2017; 55:510-518.
12. Tijet N, Patel SN, Melano RG. Detection of carbapenemase activity in Enterobacteriaceae: comparison of the carbapenem inactivation method versus the Carba NP test. *J Antimicrob Chemother* 2016; 71: 274-276.
13. Meier M, Hamprecht A. Systematic comparison of four methods for detection of carbapenemase-producing Enterobacterales directly from blood cultures. *J Clin Microbiol* 2019; 57: e00709-19.

ORIGINAL ARTICLE

Regulatory T cells in patients with lupus nephritis: relationship with disease activity

Douaa Sayed, Mona HA El-Zohri, Hala M Imam, Elbadry I Abo-Elnour, Hossam Elashmawy and Eman NasrEldin

ABSTRACT

Aims: To determine the number of $CD4^+$ $CD25^{+/high}$ T cells (Tregs), as well as Foxp3 expression, in peripheral blood mononuclear cells of systemic lupus erythematosus (SLE) patients with lupus nephritis and their relations with other activity markers and disease severity.

Methods: Forty female patients with lupus nephritis and twenty age-matched healthy female controls were enrolled. Patients fulfilled at least four criteria of SLE according to American College of Rheumatology criteria and diagnosis of lupus nephritis. The patients were classified into 2 groups based on the SLE disease activity index. The percentage and absolute number of $CD4^+$ $CD25^{+/high}$ T cells and Foxp3 expression in these cells were analysed by flow cytometry.

Results: The absolute number and percentage of Tregs ($CD4^+$ $CD25^+$ $Foxp3^+$) as well as $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells were significantly increased in patients with lupus nephritis compared to the control group. $CD4^+$ $CD25^{dim}$ $Foxp3^+$ and $CD4^+$ $CD25^-$ $Foxp3^+$ were also increased in patients with lupus nephritis. Moderate positive significant correlations were found between ESR, Anti-ds DNA and absolute number of $CD4^+CD25^-Foxp3^+$ T cells.

Conclusions: The increase in Tregs occurs in patients with active disease which confirms the immunosuppressive nature of these cells and their crucial role in maintaining the immune homeostasis against self-tissues. The $CD4^+$ T cells negative for CD25 could represent an important arm in the immunosuppression in patients with SLE and thus their role in SLE and lupus nephritis should be focused upon in the future studies.

Key words: regulatory T cells, Foxp3 expression, systemic lupus erythematosus, lupus nephritis.

N Z J Med Lab Sci 2020; 74: 76-81.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease characterised by multisystem involvement that is debilitating and can cause life-threatening organ damage (1). SLE is a chronic disease of unknown etiology with relapse and remission periods (2). Lupus nephritis is considered one of the most serious manifestations of SLE, usually arises within the early years of the disease. Lupus nephritis is histologically evident in most patients, even those without clinical manifestations of renal disease (3,4).

An imbalance in T helper type 1 (Th1/Th2) response is a major factor in SLE pathogenesis. Other T cell subsets, such as regulatory T cells (Treg cells), have also been implicated (2). $CD4^+$ $CD25^+$ Fox $p3^+$ Tregs cells play a major role in the maintenance of the immune homeostasis against self-tissues, of which all investigations should be focused upon (5). These cells prevent autoimmune or inflammatory disorders through suppressing potentially deleterious activities of Th cells. Lack or deficient function of Treg cells is responsible for many autoimmune conditions (6). Forkhead box Protein 3 (Foxp3) is a main control gene for the development as well as the function of Tregs specifically expressed on $CD4^+$ $CD25^+$ T cells (7). Fox P3 has a role in controlling the expression of IL-2 and CD25 genes in Tregs (8,9). It acts as a negative regulator of cytokine production by CD4 T cells and suppresses the transcription of IL-2 and other cytokine genes (10).

The aim of our study was to evaluate the percentages and absolute numbers of circulating T regulatory cells ($CD4^+$ $CD25^{+/high}$ T cells) and estimation of Foxp3 expression in the peripheral blood of patients with lupus nephritis to recognise its relation with different parameters, including laboratory, immunological, histopathological findings and severity of the illness.

MATERIALS AND METHODS

Study population

The present study included forty female patients with SLE who had lupus nephritis, aged 15 to 35 years (mean: 25.07 ± 5.25) from the Rheumatology outpatient clinic and inpatient wards of Internal Medicine, Assiut University Hospitals, Assiut, Egypt. Twenty apparently healthy age-matched females served as the control group, mean age 26 ± 4.8 years, with no evidence of any rheumatologic manifestations or chronic illness.

All patients fulfilled at least four criteria of SLE according to American College of Rheumatology (ACR) criteria (11), with proteinuria ≥500 mg/day and not on steroids or immunosuppressive treatment. 23 patients consented to having a renal biopsy taken. Disease activity was assessed by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (12). Criteria for the diagnosis of lupus nephritis were: (a) persistent proteinuria ≥ 0.5 g/day or > 3+; (b) cellular casts (red cell, hemoglobin, granular, tubular, or mixed); (c) elevated serum creatinine >2.0 mg %. Patients were deemed to have lupus nephritis if at least two of these criteria were present (13). Exclusion criteria were a history of viral or bacterial infection, bleeding tendency, end-stage renal disease, or if pregnant. Informed consent was obtained from all subjects and the study protocol was approved by the local ethical committee of the Faculty of Medicine, Assiut University, Assiut, Egypt.

Immunological investigations

5ml blood was collected in plain tubes, serum was separated, divided into aliquots and kept frozen at -70 C until utilised for identification of ANA, Anti ds-DNA and Anti –C1q antibodies. 5ml blood was collected into sterile EDTA-containing tubes for flow cytometric analysis.

Antinudear antibody
Detection and semi quantitation of human autoantibodies (ANA) was by an indirect fluorescent antibody assay (Kallestad, USA "Quanta flour fluorescent antibody test).

Anti-dsDNA and Anti-C1q antibodies
Quantitative estimation of IgG class autoantibodies against double-stranded DNA or C1q was by indirect solid phase enzyme immunoassay (ORGENTEC Diagnostika GmbH, Germany).

Flow cytometric analysis of T cells:
EDTA blood samples were analysed by four colour flow cytometric analysis within 24 hours of collection to quantify percentages of circulating $CD4^+$ CD25 Foxp3 T cells on a fluorescence-activated cell sorting FACS (Calibur, Becton Dickinson Immunocytometry System) using a set of fluorochrome-labeled monoclonal antibodies against Treg surface and intracellular markers (All monoclonal antibodies from e-Bioscience, USA). Isotype control antibodies were used to separate positive and negative cells. Phenotypic analysis of peripheral blood mononuclear cells (PBMC) in whole blood samples was performed for a minimum 10.000 events/tube. Expression of cell markers was assessed by the relative number of positive cells.$CD4^+$ $CD25^+$ T cells percentage specified within total $CD3^+$. $CD4^+$ $CD25^{high}$ T cells while $CD4^+$ $CD25^{dim}$T cells were specified within $CD4^+$ $CD25^+$ T cells. The absolute number of the whole $CD4^+$ was calculated. The expression of Foxp3 was determined inside the $CD4^+$ $CD25^{high,}$, $CD4^+$ $CD25^+$, $CD4^+$ $CD25^-$. $CD4^+$ $CD25^{dim}$ T cells and in the entire $CD4^+$ T cell population. $CD4^+$ $CD25^+/^{high}$ $Foxp3^+$ T cells were classified as regulatory T cells.

Renal biopsy histopathological examination
Renal biopsy specimens were fixed in 4.5% buffered formaldehyde for light microscopy. Consecutive serial 3µm thick sections were used for histological staining. Stains employed included hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), silver methenamine and Masson's trichrome. Pathological parameters, such as activity index and chronicity index, were determined by a renal pathologist using a previously reported system involving semi-quantitative scoring of specific biopsy features with minor modifications (14). Lupus nephritis was classified according to the 1995 modified World Health Organization (WHO) classification system into six different classes, from subclinical to end-stage disease *(15,*16).

Statistical analysis
Data were analysed using the Statistical Package for Social Sciences (SPSS), version 16. The t-test and Mann-Whitney U test were used to analyse quantitative parametric and non-parametric data respectively, ANOVA test was used to compare quantitative data among more than two groups, while the chi-square test was utilised for qualitative data analysis. Correlations were by linear regression analysis and Pearson correlation coefficients. Statistical significance was set at the p 0.05 level.

RESULTS

Disease activity
According to the SLE disease activity index (SLEDAI) 15 patients (37.5%) with a score of 6-10 were considered to have moderate disease activity, two patients (5%) with a score of 11-19 were considered to have moderate disease activity, while 23 patients (57.5%) with a score of ≥ 20 were considered to have very high disease activity. Patients were categorized in two main groups, Group 1 included patients with moderate disease activity while Group 2 including patients with high, or very high, disease activity. There was a significant difference in ESR, Anti-ds DNA, Anti- C1q and ANA between the two groups. There were also significant differences in disease activity parameters between the two groups and the control group except for anti-ds DNA and anti C1q antibodies levels in Group 1 patients and controls (Tables 1 and 2).

T regulatory cells
The percentage of $CD3^+$ (T lymphocytes), $CD4^+CD25^{high,}$ and $CD4^+CD25^{dim}$ (from $CD4^+CD25^+$ T cells) were significantly lower in patients with lupus nephritis compared to healthy controls. The same finding was observed by comparing group 1 and 2 with the control group while there were no significant differences between Groups 1 and 2. The absolute count of $CD3^+$ was lower in patients compared to the control group with no significant difference between Groups 1 and 2. There were significantly lower $CD4^+CD25^+$, and $CD4^+CD25^{dim}$ T cells absolute counts in patients compared to the control group. Percentages of $CD4^+$ $CD25^-$ $Foxp3^+$ and $CD4^+$ $CD25^{dim}$ $Foxp3^+$ cells were also significantly higher in patients with lupus nephritis (Table 3).

Tregs
$CD4^+$ $CD25^+$ $Foxp3^+$ and $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells percentages were significantly higher in patients while percentages were higher in patients with moderate disease activity. The number of Tregs was also higher in patients with lupus nephritis.

Histology
Class IV was the most predominant class among patients with lupus nephritis (6 out of 8 in Group1 and 8 out of 15 in Group 2). There was no significant relation between WHO class and degree of activity of disease. The activity index was increased in both groups, more in group 2 but this was not statistically significant. There was a positive relationship between the activity index and degree of activity. The chronicity index was increased in both groups, more so in Group1 than Group 2. There was an inverse relationship between the chronicity index and degree of activity of the disease. Moderate positive significant correlations were found between 1st and 2nd ESR and anti-ds DNA with absolute number of $CD4^+CD25^-$ $Foxp3^+$ T cells (Table4).

DISCUSSION

Systemic lupus erythematosus (SLE) is a chronic auto-immune illness that can affect almost every tissue. Renal involvement (lupus nephritis) has an effect on the long-term prognosis of SLE patients (1). The 5- and 10-year survival rates of lupus nephritis patients range between 83%–92% and 74–84% respectively and about 25% of the patients may develop end-stage renal failure after about 10 years from renal involvement. SLE is associated with numerous impairments in the immune system, especially regulatory functions (17). Tregs are necessary for maintaining tolerance to self-antigens by suppressing self-reactive T cells. An impaired Tregs cells function is one of the potentials that uncover the development of autoimmunity in SLE (18,19). The fundamental criteria for SLE flares include the degree of proteinuria, the disease activity index scores, ESR and complement levels. SLEDAI is a valid and reliable method to assess disease activity in SLE (12).

Our study showed that the percentage and the absolute number of $CD3^+$ and total $CD4^+$ T cells were significantly lower in patients with lupus nephritis than the control group. The decline in $CD4^+$ agrees with a large number of SLE immunological studies, as well as in other autoimmune diseases (20).

$CD4^+$ $CD25^+$ T cells play a role in the maintenance of tolerance to self-antigens and appearance of autoimmune diseases. Function and number of $CD4^+$ $CD25^+$ T cells

population in patients with SLE have been controversial (19-21). Previous results demonstrating reduced numbers of $CD4^+$ $CD25^+$ T cells, that for the most part connected with disease flares, not remission (22,23). Our study shows that the absolute number of $CD4^+$ $CD25^+$ T cells was significantly lower in peripheral blood of lupus nephritis patients than in healthy controls. Previous studies have also found diminished numbers of $CD4^+$ $CD25^+$ T cells in patients with disease flares (20, 21,24,25).

We found that the percentage of circulating $CD4^+$ $CD25^{high}$ within the population of PBMCs was significantly lower in peripheral blood of patients with lupus nephritis than in healthy controls. The fraction of $CD4^+$ $CD25^+$ T cells that express a high density of CD25 was significantly lower in patients with SLE with reduced suppressive capacity in patients with active SLE (6,23,26,27). The decline of Tregs in active SLE could be clarified by diminished production, increased destruction, by antibodies such as antilymphocyte antibodies which may target T cells, especially Tregs, and decreased peripheral expansion as evident by diminished IL-2 production and reduced responsiveness to IL-2 in lupus patients (28). The decrease in $CD4^+$ $CD25^{high}$ Tregs may be attributed to the reduced level of IL-2 generation in SLE. Diminished IL-2 prompts a decrease in Tregs populace with simultaneous autoimmunity because of excessive lymphoproliferation (29). IL-2 may not be sufficient alone to enhance Treg and require anti-CD3 for up-regulation (30). However, Sanchez *et al.* (21) found no significant difference in levels of $CD4^+$ $CD25^{high}$, $CD4^+$ $CD25^+$, $CD4^+$ $CD25^-$ and $Foxp3^+CD4^+$ T cells in SLE patients with no significant correlation between disease activity and the number of Tregs (21). Additionally, a normal suppressive potential of Tregs in SLE patients with either active or inactive disease were previously reported (20,31,32). This distinction in the outcomes could be advocated by use of high doses of steroids in that study and the greater part of patients were in an inactive disease state. Other studies clarify that the Tregs not being reduced is that they repressed the proliferation of T-effector cells to about 60% while the standard suppression assays repress nearly 95% or more. Furthermore, Lyssuk *et al.* reported a significant reduction the total number of $CD4^+$ $CD25^+$ T cells in SLE patients with lupus nephritis regardless if they started treatment with pulse cyclophosphamide or not (28). This is contradictory to a previous study by Bonelli *et al.* who did a detailed quantitative and qualitative analysis of naturally occurring $CD4^+$ $CD25^+$ Tregs from SLE patients and revealed increased proportions of $CD4^+$ $CD25^+$ Tregs among peripheral blood mononuclear cells in SLE patients (33). The most important reason for the discrepancies relied on the different definition of Tregs and the different gating strategy used. These differences could be also explained by the fact that these patients were under steroid treatment or other immunosuppressive treatment, the smaller number of patients, or could be due to the number of active cases being smaller than inactive cases (31). Those studies show that the major regulatory defect in lupus nephritis patients is in $CD4^+$ $CD25^{high}$ and $CD4^+$ $CD25^+$.

Foxp3 is more specific for gating Tregs. In our study an increase in the percentage and absolute number of Tregs which possess Foxp3 ($CD4^+$ $CD25^+FoxP3^+$ and $CD4^+$ $CD25^{high}$ $Foxp3^+$) was observed in patients with lupus nephritis but there was no significant difference in these parameters in comparing the more active group with the less active group. High Foxp3 expression in $CD4^+CD25^{high}$ T cells in SLE patients can be explained by the presence of a cofactor in $CD4^+CD25^{high}$ T cells, like B lymphocyte-induced maturation protein 1 (Blimp-1) or gene related in anergy lymphocytes (GRAIL), or as a yet unidentified molecule along with Fox P3 which could be essential for their suppressive function (34,35).

Our results are contradictory to some previous studies (18,28,36) where expression of $Foxp3^+$ was decreased in Tregs from SLE patients with active disease and poor suppressor activity. These differences could be due to the reagents used as Foxp3 can be detected by some monoclonal antibodies but not others (27). Additionally, the method of stimulation in the culture, the levels of contaminating TGF*B,* or the presence of APCs might modify the results. Other studies have shown a significant decrease in the absolute number of peripheral $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells in patients with SLE due to the presence of interferon –α and lymphocytotoxic antibodies and the level of these cells is increased after plasmapheresis, which may be due to the elimination of these antibodies.

Our study has shown no significant correlations between Tregs and different activity markers as well as histological findings. A weak negative correlation was reported previously between the absolute number of Tregs and SLEDAI (i.e. the number of Tregs decreases with increasing disease activity) (22,24,28), the proportion of $CD4^+CD25^+$ T cells and the suppressive capacity of Tregs inversely correlated with the disease activity in SLE patients (27,32).

The negative correlation between SLEDAI score and the number or percentage of Tregs in patients with the most active disease is attributed to that their Tregs had the lowest capacity to repress the T–effectors cells (30). With increased activity of the disease, as assessed by SLEDAI, there was a decrease in Tregs in the circulation of lupus nephritis patients and elevation in the titer of anti-ds-DNA antibodies. Moreover, the level of anti-ds-DNA antibodies is positively correlated with renal affection in patients with SLE. Previous result have shown that in patients who were untreated and/or newly diagnosed, the number of $CD4^+$ $CD25^+$Tregs showed an inverse correlation with the levels of anti- ds-DNA antibodies, but not with the levels of complement factors (decrease Tregs with increased activity and increase in anti- ds DNA) (32,37).

We observed that the percentage and absolute number of $CD4^+$ $CD25^-$ were higher in patients with lupus nephritis than the control group but this was not statistically significant. Increased $CD4^+$ $CD25^-$ T cells in active SLE patients may be explained by two reasons. Firstly, it might be because of the intrinsic function defect of $CD4^+$ $CD25^{high}$ cells that instantly suppresses $CD4^+$ $CD25^-$ T cell proliferation Secondly, because of increment in $CD4^+$ $CD25^-$ T cells resistance to the inhibitory effect of .$CD4^+$ $CD25^{high}$ cells (27). In contrast, Viglietta *et al.* showed a decrease in $CD4^+$ $CD25^-$ T cells in patients with SLE after allogenic stimulation of Tregs and explained this by the presence of other repressor cells that can quell $CD4^+$ $CD25^-$ T cell proliferation (38).

We found that the percentage and absolute number of $CD4^+CD25^-$ $Foxp3^+$, and $CD4^+$ $CD25^{dim}$ Foxp3 cells were higher in patients with lupus nephritis but there was no significant difference in these parameters between the more active and less active groups. Activation of $CD4^+$ $CD25^-$ T cells expressing Foxp3 prompts an increase in $Foxp^3$ with no regulatory function, the so-called ectopic expression (34,39). Another explanation of the high number and percentage of $CD4^+$ $CD25^-$ $Foxp3^+$ cells is that the function of $Foxp^3$ is not restricted to Tregs and $Foxp^3$ can be induced upon T cell receptor mediated activation. The entire population of T cells will be activated in the presence of anti-CD^3 acting as a TCR stimulant in a polyclonal fashion and heat-inactivated human serum (40).

In conclusion**,** there is strong evidence that patients with lupus nephritis have a higher number and percentage of Tregs ($CD4^+CD25^+$ $Foxp3^+$ and $CD4^+CD25^{high}$ $Foxp3^+$) in their peripheral blood. The increase in Tregs occurs in patients with active disease that confirms the immunosuppressive nature of these cells and their crucial role in maintaining the immune homeostasis against self-tissues through suppressing potentially deleterious activities of T helper cells. Previous studies have focused mainly on the role of $CD4^+$ $CD25^+$ T cells, particularly those with high expression of CD25 in SLE and lupus nephritis, but our study has shown that other cells than $CD4^+$ $CD25^+$ T cells subpopulation, namely the $CD4^+$ T cells negative for CD25 could represent an important arm in the immunosuppression in patients with SLE and thus their role in SLE and lupus nephritis should be focused upon in the future studies.

Table 1. T regulatory cells and other T cell subsets in patients with lupus nephritis.

Cells	Percentage			Absolute Count		
	Patients (n= 40)	Control (n= 20)	p	Patients (n= 40)	Controls (n= 20)	p
$CD3^+$	18.73 ± 2.29	29.59 ± 2.97	0.001	1.40 ± 0.19	2.02 ± 0.22	0.007
$CD4^+CD25^+$	1.52 ± 0.29	0.72 ± 0.15	0.447	1.10 ± 0.14	1.74 ± 0.20	0.005
$CD4^+CD25^{high}$	15.92 ± 1.55	24.60 ± 1.99	0.001	0.12 ± 0.03	0.05 ± 0.01	0.599
$CD4^+CD25^{dim}$	15.00 ± 1.68	23.18 ± 1.92	0.001	0.97 ± 0.12	1.64 ± 0.19	0.004
$CD4^+CD25^-$	13.07 ± 2.52	7.19 ± 0.78	0.240	0.79 ± 0.15	0.53 ± 0.07	0.718
$CD4^+CD25^-Foxp3^+$	5.55 ± 2.23	0.72 ± 0.25	0.004	0.03 ± 0.01	0.00 ± 0.00	0.002
$CD4^+CD25^{dim}Foxp3^+$	9.85 ± 2.18	3.12 ± 0.95	0.002	0.08 ± 0.02	0.04 ± 0.01	0.297
Tregs:						
$CD4+CD25^+Foxp3^+$	19.68 ± 2.50	9.34 ± 1.79	0.002	0.18 ± 0.03	0.14 ± 0.02	0.931
$CD4+CD25^{high}Foxp3^+$	61.25 ± 4.75	43.19 ± 6.12	0.031	0.06 ± 0.02	0.02 ± 0.00	0.270

Results are mean ± SEM. CD = cluster of differentiation. Foxp3 = forked head box protein 3. Tregs = regulatory T cells.

Table 2. T regulatory cells and other T cell subsets in different groups of patients with lupus nephritis and controls.

Cells		Patients		Controls	p^1	p^2	p^3
		Group 1 (n= 15)	Group 2 (n= 25)	(n= 20)			
$CD3^+$	Count	1.30 ± 0.27	1.46 ± 0.26	2.02 ± 0.22	0.967	0.005	0.043
	%	15.86 ± 2.90	20.46 ± 3.22	29.59 ± 2.97	0.394	0.000	0.015
$CD4^+CD25^+$	Count	1.10 ± 0.23	1.10 ± 0.19	1.74 ± 0.20	0.989	0.042	0.008
	%	1.61 ± 0.51	1.46 ± 0.34	0.72 ± 0.15	0.665	0.404	0.591
$CD4^+CD25^{high}$	Count	0.09 ± 0.03	0.14 ± 0.05	0.05 ± 0.01	0.911	0.559	0.714
	%	17.27 ± 2.90	15.10 ± 1.79	24.60 ± 1.99	0.655	0.033	0.000
$CD4^+CD25^{dim}$	Count	0.99 ± 0.20	0.95 ± 0.15	1.64 ± 0.19	0.989	0.030	0.007
	%	16.01 ± 2.68	14.39 ± 2.18	23.18 ± 1.92	0.485	0.033	0.001
$CD4^+CD25^-$	Count	0.71 ± 0.27	0.84 ± 0.18	0.53 ± 0.07	0.357	0.677	0.417
	%	13.90 ± 5.09	12.57 ± 2.73	7.19 ± 0.78	0.379	0.764	0.132
$CD4^+CD25^-Foxp3^+$	Count	0.04 ± 0.02	0.02 ± 0.01	0.00 ± 0.00	0.390	0.052	0.002
	%	9.61 ± 5.81	3.12 ± 0.64	0.72 ± 0.25	0.714	0.115	0.002
$CD4^+CD25^{dim}Foxp3^+$	Count	0.06 ± 0.02	0.08 ± 0.02	0.04 ± 0.01	0.476	0.868	0.160
	%	10.12 ± 5.14	9.69 ± 1.76	3.12 ± 0.95	0.235	0.055	0.002
Tregs:							
$CD4^+CD25^{+-}Foxp3^+$	Count	0.18 ± 0.06	0.18 ± 0.04	0.14 ± 0.02	0.989	0.907	0.837
	%	20.85 ± 5.05	18.97 ± 2.71	9.34 ± 1.79	0.989	0.015	0.004
$CD4^+CD25^{high}Foxp3^+$	Count	0.05 ± 0.02	0.07 ± 0.03	0.02 ± 0.00	0.308	0.037	0.809
	%	65.92 ± 7.97	58.72 ± 5.98	43.19 ± 6.12	0.373	0.031	0.091

Results are mean ± SEM. CD = cluster of differentiation. Foxp3 = forked head box protein 3. Tregs =regulatory T cells. [1]Comparison between Group 1 and Group 2. [2]Comparison between Group 1 and controls. [3]Comparison between Group 2 and controls.

Table 3. Histological results of lupus nephritis in SLE patients according to the 1995 modified WHO classification.

	All studied patients n= 23 n (%)	Group 2 n= 15 n (%)	Group 1 n= 8 n (%)
Class I	0 (0%)	-	-
Class II	3 (13.0%)	2 (13.3%)	1 (12.5%)
Class III	5 (21.7%)	4 (26.7%)	1 (12.5%)
Class IV	14 (60.9%)	8 (53.3%)	6 (75.0%)
Class V	1 (4.3%)	1 (6.7%)	0 (0%)
Activity index \24 score (Mean ± SD)	6.30 ± 3.59	7.33 ± 3.92	4.38 ± 1.77
Chronicity index \12 score (Mean ± SD)	4.13 ± 2.07	3.60 ± 2.13	5.12 ± 1.64

Table 4. Correlations between T regulatory cells and different parameters in patients with lupus nephritis.

	1st ESR		2nd ESR		24h urine protein		Anti-ds DNA		Anti C1q (U\mL)		SLEDAI		WHO class	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Percentage:														
- Tregs:														
$CD4^+$ $CD25^+$ $Foxp3^+$	-0.010	0.952	0.010	0.950	-0.11	0.5	-0.196	0.226	-0.125	0.443	0	0.999	-0.381	0.073
$CD4^+$ $CD25^{high}$ $Foxp3^+$	-0.248	0.140	-0.314	0.058	0.001	0.997	-0.155	0.360	-0.237	0.158	-0.212	0.208	-0.109	0.638
- $CD4^+CD25^{dim}$ $Foxp3^+$	0.126	0.438	0.168	0.301	-0.021	0.899	0.022	0.893	0.046	0.776	0.197	0.223	-0.352	0.100
- $CD4^+$ $CD25^-$ $Foxp3^+$	0.069	0.672	0.096	0.554	-0.145	0.372	0.068	0.679	-0.108	0.508	0.056	0.731	0.008	0.971
Absolute count:														
- Tregs:														
$CD4^+$ $CD25^+$ $Foxp3^+$	0.026	0.843	0.029	0.829	-0.04	0.805	-0.052	0.694	-0.081	0.538	-0.006	0.972	0.092	0.676
$CD4^+$ $CD25^{high}$ $Foxp3^+$	0.060	0.663	0.063	0.648	0.014	0.934	-0.113	0.411	-0.046	0.736	-0.009	0.958	0.110	0.634
- $CD4^+CD25^{dim}$ $Foxp3^+$	0.155	0.238	0.157	0.230	-0.076	0.641	0.058	0.662	0.037	0.780	0.161	0.32	0.119	0.588
- $CD4^+$ $CD25^-$ $Foxp3^+$	0.372	0.003*	0.385	0.002*	-0.055	0.734	0.280	0.030*	0.158	0.228	0.107	0.513	0.125	0.570

Tregs: regulatory T cells.

AUTHOR INFORMATION

Douaa Sayed, MD, Professor[1]
Mona HA El-Zohri, MD[2]
Hala M Imam, MD, Professor[2]
Elbadry I Abo-Elnour, MD, Professor[2]
Hossam Elashmawy, MD, Lecturer[3]
Eman NasrEldin, MD, Assistant Professor[4]

[1]Department of Clinical Pathology, South Egypt Cancer Institute, Assiut, Egypt
[2]Department of Internal Medicine, Faculty of Medicine, Assiut University, Egypt
[3]Department of Clinical Pathology, Faculty of Medicine, Al-Azhar University, Assiut, Egypt
[4]Department of Clinical Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt

Corresponding author: Dr. Eman NasrEldin.
Email: emannasr2000@yahoo.com

REFERENCES

1. Stratigou V, Doyle AF, Carlucci F, Stephens L, Foschi V, Castelli M, et al. Altered expression of signaling lymphocyte activation molecule receptors in T-cells from lupus nephritis patients-a potential biomarker of disease activity *Rheumatology (Oxford)* 2017; 56: 1206-1216.
2. Mesquita D Jr, Kirsztajn GM, Franco MF, Reis LA, Perazzio SF, Mesquita FV, et al. CD4+ T helper cells and regulatory T cells in active lupus nephritis: an imbalance towards a predominant Th1 response? *Clin Exp Immunol* 2018; 191: 50-59.
3. D'Agati VS, Appel GB. Lupus nephritis: pathology and pathogenesis. In: DJ Wallace and BH Hahn, eds. Dubois' Lupus Erythematosus, 7th Edition, 2006. Lippincott Williams & Wilkins, Philadelphia, PA: 1094-1111.
4. Rose BD, Appel GB, Schur PH. Types of renal disease in systemic lupus erythematosus. In: UpToDate, 2011. Walters Kluwer, Philadelphia, USA. http://www.uptodate.com
5. Sakaguchi S. Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004; 22: 531-562.

6. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF down-modulates the function of human $CD4^+$ $CD25^{high}$ T-regulatory cells. *Blood 2006;* 108: 253-261.
7. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in $CD4^+$ $CD25^+$ T regulatory cells. *Nat Immunol* 2003; 4: 337-342.
8. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^+$ $CD25^+$ regulatory T cells. *Nat Immunol* 2003; 4: 330-336.
9. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; 299: 1057-1061.
10. Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *J Biol Chem* 2001; 276: 37672-37679.
11. Petri M, Orbai AM, Alarcón GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for systemic lupus erythematosus. *Arthritis Rheum* 2012; 64: 2677-2686.
12. Reichlin M. Serological correlations with nephritis in systemic lupus erythematosus. *Clin Immunol* 2005; 117: 12-14.
13. Austin HA 3rd, Boumpas DT, Vaughan EM, Balow JE. Predicting renal outcomes in severe lupus nephritis: contributions of clinical and histologic data. *Kidney Int* 1994; 45: 544–550.
14. Churg J, Bernstein J, Glassock RJ. Renal Disease: Classification and Atlas of Glomerular Disease, 2nd ed., 1995. Igaku-Shoin Medical Pub, New York: 151-156.
15. Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, Alpers CE, Appel GB, et al. The classification of glomerulonephritis in systemic lupus erythematosus revisited. *J Am Soc Nephrol 2004;* 15: 241-250. [Erratum in: *J Am Soc Nephrol* 2004; 15: 835-836].
16. Lieberman LA, Tsokos GC. The IL-2 defect in systemic lupus erythematosus disease has an epansive effect on host immunity. *J Biomed Biotechnol* 2010; 2010: 740619.
17. Lim HW, Hillsamer P, Kim CH. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J Clin Invest 2004;* 114: 1640-1649. [Erratum in: *J Clin Invest* 2005; 115:195].
18. Liu MF, Wang CR, Fung LL, Wu CR. Decreased $CD4^+$ $CD25^+$ T cells in peripheral blood of patients with systemic lupus erythematosus. *Scan J Immunol* 2004; 59: 198-202.
19. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, et al. Global natural regulatory T cell depletion in active systemic lupus erythematosus. *J Immunol* 2005; 175: 8392-8400.
20. Alvarado-Sánchez B, Hernández-Castro B, Portales-Pérez D, Baranda,L, Layseca-Espinosa E, Abud-Mendoza C, et al. Regulatory T cells in patients with systemic lupus erythematosus. *J Autoimmun 2006;* 27: 110-118.
21. Lee JH, Wang LC, Lin YT, Yang YH, Lin DT, Chiang BL. Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in paediatric patients with systemic lupus erythematosus. *Immunology* 2006; 117: 280-286.
22. Bonelli M, von Dalwigk K, Savitskaya A, Smolen JS, Scheinecker C. Foxp3 expression in $CD4^+$ T cells of patients with systemic lupus erythematosus: a comparative phenotypic analysis. *Ann Rheum Dis* 2008; 67: 664-671.
23. Fathy A, Mohamed RW, Tawfik GA, Omar AS. Diminished $CD4^+$ $CD25^+$ T- lymphocytes in peripheral blood of patients with systemic lupus erythematosus. *J Immunol* 2006; 12: 25-31.
24. Shevach EM. $CD4^+$ $CD25^+$ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002; 2: 389–400.
25. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. $CD4^+$ $CD25^{high}$ regulatory cells in human peripheral blood. *J Immunol 2001:* 167: 1245-1253.
26. Valencia X, Yarboro C, Illei G, Lipsky PE. Deficient $CD4^+$ $CD25^{high}$ T regulatory cell function in patients with active systemic lupus erythematosus. *J Immunol* 2007; 178: 2579-2588.
27. Lyssuk EY, Torgashina AV, Soloviev SK, Nassonov EL, Bykovskaia, SN. Reduced number and function of $CD4^+$CD25highFoxP3 regulatory T cells in patients with systemic lupus erythematosus. *Adv Exp Med Biol* 2007; 601: 113–119.
28. Golding A. SLE and Tregs: what we know and what we would like to know. Johns Hopkins University School of Medicine, 2012.
29. Skapenko A, Kalden JR, Lipsky PE, Schulze-Koops H. The IL-4 receptor α-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing $CD25^+CD4^+$ regulatory T cells from $CD25^-$ $CD4^+$ precursors. *J Immunol 2005;* 175: 6107-6116.
30. Yan B, Ye S, Chen G, Kuang M, Shen N, Chen S. Dysfunctional $CD4^+$,$CD25^+$ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-alpha-producing antigen-presenting cells. *Arthritis Rheum* 2008; 58: 801–812.
31. Lee HY, Hong YK, Yun, HJ. Altered frequency and migration capacity of $CD4^+CD25^+$ regulatory T cells in systemic lupus erythematosus. *Rheumatology (Oxford)* 2008; 47: 789–794.
32. Bonelli M, Savitskaya, A, Von Dalwigk K, Steiner CW, Aletaha,D, Smolen JS. et al. Quantitative and qualitative deficiencies of regulatory T cells in patients with systemic lupus erythematosus (SLE). *Int Immunol* 2008; 20: 861-868.
33. Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A, et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A* 2006; 103: 6659-6664.
34. Martins GA, Cimmino L, Shapiro-Shelef M, Szabolcs M, Herron A, Magnusdottir E, et al. Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat Immunol 2006;* 7: 457-465.
35. Baráth S, Soltész P, Kiss E, Aleksza M, Zeher M, Szegedi G, et al. The severity of systemic lupus erythematosus negatively correlates with the increasing number of $CD4^+CD25^{high}$Fox $P3^+$ regulatory T cells during repeated plasmapheresis treatments of patients. *Autoimmunity* 2007; 40: 521-528.
36. Ponchel F, Vital E, Kingsbury SR, El-Sherbiny YM. CD4+T-cell subsets in rheumatoid arthritis. *Int J Clin Rheumatol* 2012; 7: 37-53.
37. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by $CD4^+CD25^+$ regulatory T cells in patients with multiple sclerosis. *J Exp Med* 2004; 199: 971-979.
38. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human $CD4^+CD25^-$ T cells. *J Clin Invest 2003;* 112: 1437-1443.
39. Allan SE, Passerini L, Bacchetta R, Crellin N, Dai M, Orban PC, et al. The role of 2 FOXP3 isoforms in the generation of human $CD4^+$ Tregs. *J Clin Invest* 2005; 115: 3276-3284.

COVID-19 TESTING SOLUTIONS

ELISA TEST KITS

DIA.PRO

COVID-19 IgM

Monitoring of the immune response to COVID-19, in particular in the early stage of the infection as its markers in addition to PCR.

COVID-19 IgG

Monitoring of the immune response to COVID-19. Recommended in particular for testing:

(a) **health care workers** at risk of COVID-19 infection to verify whether or not they might be naturally protected;

(b) **patients** recovered from the infection, negative for COVID-19 PCR, to assure they developed IgG antibodies and are protected, confirming a full recover from the infection.

COVID-19 IgG Confirmation

Confirmation/typing of human samples screened as positive for IgG antibodies to COVID-19. The test is aimed to identify the specificity of antibodies to the major immunodominant COVID-19 antigens Nucleocapsid, Spike 1 and Spike 2.

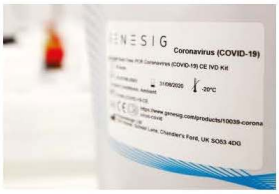
COVID-19 IgA

Monitoring of the immune response to COVID-19, in particular in the early stage of the infection as its marker in addition to IgG assay and PCR.

REAL-TIME PCR

Coronavirus (COVID-19) CE IVD Real-Time PCR Kit

- Rapid detection and exclusive to the COVID-19 strain
- Does not detect other related coronavirus strains
- High priming efficiency
- Accurate controls to confirm extraction, and assay validity
- Lyophilised components for ambient shipping
- Highly specific detection profile



genesig q32 Real-Time PCR Instrument

Providing Fast Results with genesig Kits

- Guaranteed results using 550 genesig kits
- Fast DNA and RNA analysis in less than 60 minutes
- Quick and easy set-up and run
- Automated data analysis result calling
- Small and robust instrument
- Exceptional value for money

AUTOMATED MICROPLATE INSTRUMENTATION

DYNEX® TECHNOLOGIES

Perpetual Innovations for Smarter, Efficient & Reliable Detection

DS2®

The innovative DS2® is designed for clinical, research and industrial labs that want to enhance their processes through automation. Installed in over 3,500 labs worldwide, the DS2® is the solution for the rigorous, repeatable analyses required in critical applications including immunology, infectious disease, hormone, allergy, blood-chemistry, drugs-of-abuse testing, food safety, forensics, and environmental testing among others.

DSX®

The DSX® delivers everything needed to ensure the rigorous, repeatable analyses required in critical applications including immunology, infectious disease, hormone, allergy, blood-chemistry, drugs-of-abuse testing, food safety, forensics and environmental testing.

AGILITY®

AGILITY® is redefining ELISA with ingenious design, total automation and a revolutionary approach to reagent loading that boosts productivity and minimises hands-on time like never before. For true walk-away freedom, nothing beats AGILITY®.

These ELISA and RT-PCR kits are suitable for use on various other existing instrument platforms. Please contact us to seek confirmation. All kits are CE and IVD marked.

Contact **Mediscope International** your exclusive distributor of Dynex Technologies on **0800 840 010** or **email info@mediscope.co.nz** to learn more about the DYNEX® instrumentations. Remember to ask us about our extensive range ELISA test kits that can help you with your research and/or routine laboratory work.

mediscope
Empowering Healthcare and Science

0800 840 010
www.mediscope.co.nz
info@mediscope.co.nz

ORIGINAL ARTICLE

Lipid ratios are not good predictors of insulin resistance among healthy adults from Southwest Nigeria

Esther Ngozi Adejumo, Adedeji Olusola Adejumo and Omobolanle Abioye Ogundahunsi

ABSTRACT

Background: Lipid ratios are useful biomarkers of insulin resistance in Caucasians. This study assessed the predictive ability of triglyceride and lipid ratios in predicting insulin resistance among healthy adults in Southwest Nigeria.
Methods: The study was a descriptive cross-sectional. The homeostasis model assessment for insulin resistance (HOMA- IR) was used to assess insulin resistance in healthy volunteers. The Receiver Operative Characteristics (AUC) curve was used to assess the discriminative abilities of triglyceride, the ratio of triglyceride, total cholesterol and low-density lipoprotein cholesterol (LDLc) to high-density lipoprotein cholesterol (HDLc) triglyceride/ HDLc ratio, cholesterol / HDLc ratio and LDLc / HDLc ratio respectively to discriminate insulin resistance.
Results: Five hundred and twenty healthy adults were enrolled. The mean age of the participants was 46.7±14.6 years, 72.7% were females. There were no differences in mean age, total cholesterol, LDLc, triglycerides, HDLc, cholesterol/HDLc ratio, triglyceride /HDLc ratio, LDLc/HDLc ratio and HOMA-IR ($p>0.05$). The triglyceride /HDLc ratio had the largest AUC in men (0.591, 95% CI: 0.491 – 0.685; $p= 0.122$) compared to women (0.644, 95% CI: 0.586 – 0.703; $p <0.001$).
Conclusion: Lipid ratios and triglycerides are not useful in predicting insulin resistance in men and women in Southwest Nigeria.
Keywords: Lipid ratios, insulin resistance, Nigeria.

N Z J Med Lab Sci 2020; 74: 83-86.

INTRODUCTION

The relationship of insulin resistance with obesity and cardio-metabolic diseases have long been established (1,2). Conventionally, triglyceride and high-density lipoprotein cholesterol levels (HDLc) are elevated and reduced respectively in individuals with insulin resistance (1). The current methods used for assessment of insulin resistance have inherent shortcomings. The direct methods, the hyperinsulinaemic-euglycemic clamp test is not useful in clinical application and epidemiological investigation because it is costly, has complicated application, the method is invasive and time-consuming (3,4). The lack of a standardised method for insulin estimation is a major drawback in the clinical utility of the homeostasis model assessment of insulin resistance (HOMA-IR) (the indirect method) ([5).

Recently, plasma lipids have been suggested to be simpler and more predictive for early identification of patients with insulin resistance at no additional cost. Notable is the ratio of triglycerides, total cholesterol and low-density lipoprotein cholesterol (LDLc) to HDLc: triglyceride/HDLc, cholesterol/ HDLc and LDLc/HDLc ratios, respectively (6-8).

Previous studies have identified the triglyceride /HDLc ratio as a useful biomarker of insulin resistance (9,10), diabetes (11) and coronary heart diseases (12). There is also an association of insulin resistance with the LDLc/HDLc and cholesterol/HDLc ratios (12,13). Crucially, there are ethnic (6,10,14) and gender (15) variations in the relationship between insulin resistance and the triglyceride /HDLc ratio. The appropriateness of the triglyceride /HDLc ratio in some populations, such as Taiwanese (8), Chinese (16,17), Korean (15) and Indian (18), have been reported.

Very few studies have assessed the ability of lipid ratios to predict insulin resistance in Nigeria. A previous study from Southeastern Nigeria suggested that lipid ratios were not sensitive markers of insulin resistance (19). The relatively small sample size and involvement of only women in that study were a limitation and further studies are warranted. This present study assessed the usefulness of lipid ratios as markers of insulin resistance in Southwest Nigeria and is a follow up to our initial study that determined the prevalence of metabolic syndrome in two communities in Southwest Nigeria using three different definitions (20)

METHODS

Study design and subjects

This was a descriptive cross-sectional study. Healthy volunteers were consecutively recruited through a medical outreach conducted in a rural and urban community in two Southwest states of Nigeria. The two communities were selected using multi-staged sampling methods. Information on social, demographic and clinical details, including gender, age, cigarette intake, alcohol intake, blood pressure and height and weight measurements were obtained using a structured questionnaire. Other details of the study population and sampling were earlier published (20). Ethical approvals were obtained from the Institution Review Boards of the Lagos State University Teaching Hospital Lagos and Babcock University Ogun State. Participants gave written informed consent.

Biochemical assessment

Venous blood was collected from participants after an overnight fast (8 – 12 hours) for the determination of fasting plasma glucose, lipids, and serum insulin. Enzymatic methods (Randox Laboratories Ltd,, United Kingdom) were used to measure fasting plasma glucose, total cholesterol, triglycerides and HDLc, while LDLc was calculated using the Friedewald equation (21) The triglyceride /HDLc, cholesterol/ HDLc and LDLc/HDLc ratios were also calculated. An enzyme-linked immunosorbent assay (DRG Instruments, GmbH, Germany) was used to measure fasting serum insulin.

Determination of insulin resistance

We used the HOMA- IR method (fasting serum insulin (mU/L) X fasting glucose (mmol/l) / 22.5) to assess insulin resistance and a HOMA-IR cutoff ≥ 2 was used to define insulin resistance (22).

Data analysis

The Statistical Package for Social Sciences (SPSS) IBM version 22 was used for data analysis. The means and standard deviations of continuous variables were calculated while categorical variables were presented as frequencies and percentages. The means of triglycerides and lipid ratios of independent groups were compared using the Student 't' test (2 tailed). The correlation of HOMA-IR (used as a continuous variable) with triglycerides and lipid ratios were assessed by Pearson's correlation. The ability of triglycerides and lipid ratios to discriminate Metabolic syndrome was determined with the receiver operative characteristics (ROC) curve, the diagnostic ability of triglycerides and the lipid ratios are proportional to the area under the curve (AUC). An AUC of 1.0 indicates perfect discrimination (23).

RESULTS

A total of 520 healthy adults participated in the study. The mean age was 46.7±14.6 years while the number of females were more than males with no gender difference in the mean age, total cholesterol, LDLc, TRG, HDLc, total cholesterol/HDLc ratio, triglyceride/HDLc ratio, LDLc/HDLc ratio or HOMA-IR (Table 1).

A total of 226 (43.5%) of the participants had insulin resistance. Compare to individuals without insulin resistance, participants with insulin resistance had a higher mean age, triglycerides and cholesterol/HDLc and TRG/HDLc ratio. There was no association of mean cholesterol/LDLc and LDLc/HDLc ratios with insulin resistance while HDLc levels were lower in participants with insulin resistance (Table 2).

There was no correlation of total cholesterol, LDLc, cholesterol/HDLc ratio or LDLc/ HDLc ratio with HOMA-IR. Triglycerides and the triglyceride/HDLc ratio were positively correlated with HOMA-IR while HDLc was negatively correlated with HOMA-IR (Table 3). Table 4 shows the AUC of lipid ratios and triglycerides discriminating insulin resistance in men and women. The triglyceride /HDLc ratio had the largest AUC in men, compared to women.

Table 1. Clinical parameters of study population by gender.

Variable	Whole Population (n = 520)	Male n = 142	Female n = 378	p
Age (years)	46.7±14.6	46.1±14.5	47.0±14.6	0.535
SBP (mm/Hg)	125.8±23.8	127.0±20.4	125.4±24.9	0.505
DBP (mm/Hg)	77.9±13.1	77.9±12.5	77.9±13.3	0.954
Total cholesterol (mg/dL)	175.5±53.9	173.4±54.0	176.4±53.9	0.572
LDLc (mg/dL)	102.5±54	102.4±56.3	102.6±53.5	0.980
Triglycerides (mg/dL)	90.8±56.1	92.8±69.8	90.1±50.1	0.619
HDLc (mg/dL)	54.8±20.4	52.4±19.0	55.8±20.8	0.091
HOMA-IR	2.1±1.1	2.2±1.1	2.1±1.1	0.404
Total cholesterol/HDLc ratio	3.7±1.9	3.8±1.9	3.6±1.9	0.384
Triglyceride/HDLc ratio	1.9±1.6	2.0±1.9	1.9±1.5	0.337
LDLc/HDLc ratio	2.3±1.7	2.4±1.8	2.3±1.7	0.412

SBP = systolic blood pressure, DBP = diastolic blood pressure, HDLc = high density lipoprotein cholesterol, LDLc= low density lipoprotein cholesterol.

Table 2. Association of lipid and lipid ratios with insulin resistance.

Variable	Insulin resistance		p
	Present (n = 226)	Absent (n = 204)	
Age (years)	48.2±13.6	45.6±15.3	0.048
SBP (mm/Hg)	131.5±22.7	121.4±23.7	<0.001
DBP (mm/Hg)	81.0±13.0	75.6±12.7	<0.001
Total cholesterol (mg/dL)	170.8±52.3	179.2±54.9	0.080
LDLc (mg/dL)	99.8±52.1	104.6±55.9	0.327
Triglycerides (mg/dL)	100.5±69.5	83.4±41.8	0.001
HDLc (mg/dL)	50.9±19.1	57.9±20.9	<0.001
Total cholesterol/HDLc ratio	3.9±2.1	3.5±1.8	0.046
Triglyceride/HDLc ratio	2.3±2.1	1.6±1.1	<0.001
LDLc/HDLc ratio	2.4±1.8	2.2±1.7	0.188

SBP = systolic blood pressure, DBP = diastolic blood pressure, HDLc = high density lipoprotein cholesterol, LDLc = low density lipoprotein cholesterol.

Table 3. Correlation of insulin and insulin resistance with lipids and lipid ratios.

Variable	Insulin resistance (HOMA-IR) r	p
Total cholesterol (mg/dL)	-0.027	0.543
Triglycerides (mg/dL)	0.260	<0.001
HDLc (mg/dL)	-0.130	0.004
LDLc (mg/dL)	-0.033	0.452
Total cholesterol/HDLc ratio	0.054	0.221
Triglyceride/HDLc ratio	0.225	<0.001
LDLc/HDLc ratio	0.017	0.700

HDLc = high density lipoprotein cholesterol, LDLc= low density lipoprotein cholesterol.

Table 4: Area under the receiver operative curve of lipid ratios which best predicts insulin resistance.

Variable	AUROC	95%CI	p
Men			
Triglycerides (mg/dL)	0.581	0.487 – 0.674	0.211
Total cholesterol/HDLc ratio	0.490	0.396 – 0.585	0.386
Triglyceride/HDLc ratio	0.591	0.497 – 0.685	0.122
LDLc/HDLc ratio	0.475	0.380 – 0.569	0.258
Women			
Triglycerides (mg/dL)	0.581	0.520 – 0.642	0.036
Total cholesterol/HDLc ratio	0.579	0.520 – 0.638	0.003
Triglyceride/ HDLc ratio	0.644	0.586 – 0.703	<0.001
LDLc/HDLc ratio	0.551	0.491 – 0.612	0.027

AUROC = area under the receiver operative curve, HDLc = high density lipoprotein cholesterol, LDLc= low density lipoprotein cholesterol.

DISCUSSION

Insulin resistance is characteristic of type 2 diabetes and its important link to the pathophysiology of metabolic syndrome and cardiovascular disease is well known. Interestingly, lipids and lipoprotein ratios have been used to discriminate insulin resistance because it is believed to offer an alternative approach to the hyperinsulinaemic-euglycemic clamp (24). There are gender and race variations in the ability of the triglyceride/ HDLc ratio to discriminate insulin resistance (25).

Although the triglyceride/HDLc ratio had the largest AUC in men in our study, it was not predictive of insulin resistance in men and only weakly discriminatory in women. Our findings are similar to what has been reported in a Nigerian study (19). Though there were no gender differences in the HDLc levels in our study, HDLc levels are higher in women than men which warrant the recognition of the sex-specific difference in lipid profile to avoid misleading conclusions (26).

Studies among black women from United States, Southern and Western Africa (27) and other studies among African Americans, Hispanics and South Asians suggested that triglycerides and the triglyceride/HDLc ratio are not useful biomarkers of insulin resistance in blacks (6,9,10). On the contrary, studies among Chinese, Mexicans Americans and non-Hispanics whites and blacks suggested that triglycerides and the triglyceride/HDLc ratio were good predictors of insulin resistance (17,25). The pattern of dyslipidemia is not similar in Caucasians and Africans. In Caucasians, dyslipidemia is typified by high triglycerides and low HDLc, but triglycerides and HDLc levels respectively are normal and low in Africans (27,28). It may also explain the genetic differences in lipid metabolism between Caucasians and Blacks (19).

A study of 2000 African Americans showed that the triglyceride / HDLc ratio was able to predict insulin resistance only in men (26). A study based on black and white women reported the triglyceride/HDLc ratio as a predictor of insulin resistance among white women but not among black women from Southern and Western Africa, and the United States (27).

In conclusion, lipid ratios and triglycerides are not useful in discriminating insulin resistance in men and women in Southwest Nigeria. Though studies among Caucasians have shown that the triglyceride/HDLc ratio to be a good predictor of insulin resistance, there is little evidence to support this claim among Africans. Further studies are needed to ascertain useful biomarkers of insulin resistance, especially in Africans.

AUTHOR INFORMATION

Esther Ngozi Adejumo, PhD, Senior Lecturer[1]
Adedeji Olusola Adejumo, MPH FMCPH, Public Health Consultant[2]
Omobolanle Abioye Ogundahunsi, PhD, Professor[3]

[1]Department of Medical Laboratory Science, Babcock University, Ilishan-Remo, Ogun State, Nigeria
[2]Department of Community Health and Primary Health Care, Lagos State University Teaching Hospital, Ikeja, Lagos State, Nigeria
[3]Department of Chemical Pathology, Olabisi Onabanjo University, Ogun State, Nigeria

Correspondence: Esther Ngozi Adejumo.
Email: adejumoe@babcock.edu.ng

REFERENCES

1. Reaven GM. Banting Lecture 1988: role of insulin resistance in human disease. *Diabetes* 1988; 37:1595–1607.
2. Xia C, Li R, Zhang S, Gong L, Ren W, Wang Z, et al. Lipid accumulation product is a powerful index for recognizing insulin resistance in nondiabetic individuals. *Eur J Clin Nutr* 2012; 66:1035–1038.
3. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 1979; 237: E214–F223.
4. Keskin M, Kurtoglu S, Kendirci M, Atabek ME, Yazici C. Homeostasis model assessment is more reliable than the fasting glucose/insulin ratio and quantitative insulin sensitivity check index for assessing insulin resistance among obese children and adolescents. *Pediatrics* 2005;115: e500–3.
5. Miller WG, Thienpont LM, Van Uytfanghe K, Clark PM, Lindstedt P, Nilsson G, et al. Toward standardization of insulin immunoassays. *Clin Chem* 2009; 55:1011–1018.
6. Kim-Dorner SJ, Deuster PA, Zeno SA, Remaley AT, Poth M. Should triglycerides and the triglycerides to high-density lipoprotein cholesterol ratio be used as surrogates for insulin resistance? *Metabolism* 2010; 59: 299–304.
7. Kawamoto R, Tabara Y, Kohara K, Miki T, Kusunoki T, Takayama S, et al. Low densitylipoprotein cholesterol to high-density lipoprotein cholesterol ratio is the best surrogate marker for insulin resistance in non-obese Japanese adults. *Lipids Health Dis* 2010; 9:138.
8. Yeh WC, Tsao YC, Li WC, Tzeng IS, Chen LS, Chen JY. Elevated triglyceride-to-HDL cholesterol ratio is an indicator for insulin resistance in middle-aged and elderly Taiwanese population: a cross-sectional study. *Lipids Health Dis* 2019; 18:176.
9. Giannini C, Santoro N, Caprio S, Kim G, Lartaud D, Shaw M, et al. The triglyceride-to-HDL cholesterol ratio: association with insulin resistance in obese youths of different ethnic backgrounds. *Diabetes Care* 2011; 34:1869–74.
10. Mostafa SA, Davies MJ, Morris DH, Yates T, Srinivasan BT, Webb D, et al. The association of the triglyceride- to-HDL cholesterol ratio with insulin resistance in White European and South Asian men and women. *PLoS One* 2012; 7: e50931.
11. Hadaegh F, Hatami M, Tohidi M, Sarbakhsh P, Saadat N, Azizi F. Lipid ratios and appropriate cut off values for prediction of diabetes: a cohort of Iranian men and women. *Lipids Health Dis* 2010; 9: 85.
12. Kannel WB, Vasan RS, Keyes MJ, Sullivan LM, Robins SJ. Usefulness of the triglyceride–high-density lipoprotein versus the cholesterol–high-density lipoprotein ratio for predicting insulin resistance and cardiometabolic risk (from the Framingham Offspring Cohort). *Am J Cardiol* 2008; 101: 497–501.
13. Kawamoto R, Tabara Y, Kohara K, Miki T, Kusunoki T, Takayama S, et al. Low-density lipoprotein cholesterol to high-density lipoprotein cholesterol ratio is the best surrogate marker for insulin resistance in non-obese Japanese adults. *Lipids Health Dis* 2010; 9: 138.
14. Gasevic D, Frohlich J, Mancini GB, Lear SA. The association between triglyceride to high-density-lipoprotein cholesterol ratio and insulin resistance in a multiethnic primary prevention cohort. *Metabolism* 2012; 61: 583–589.
15. Byun AR, Lee SW, Lee HS, Shim KW. What is the most appropriate lipid profile ratio predictor for insulin resistance in each sex? A cross-sectional study in Korean populations (The Fifth Korea National Health and Nutrition Examination Survey). *Diabetol Metab Syndr* 2015; 7: 59.
16. Zhang L, Chen S, Deng A, Liu X, Liang Y, Shao X, et al. Association between lipid ratios and insulin resistance in a Chinese population. *PLoS ONE* 2015; 10: e0116110.
17. Chiang JK, Lai NS, Chang JK, Koo M. Predicting insulin resistance using the triglyceride-to-high density lipoprotein cholesterol ratio in Taiwanese adults. *Cardiovasc Diabetol* 2011; 10: 93.
18. Ray S, Talukdar A, Sonthalia N, Saha M, Kundu S, Khanra D, et al. Serum lipoprotein ratios as markers of insulin resistance: A study among non-diabetic acute coronary syndrome patients with impaired fasting glucose. *Indian J Med Res* 2015; 141: 62–67.
19. Young, EE, Okafor CN, Iroezindu MO, Agbalu IS. Insulin resistance, metabolic syndrome, and lipids in African women. *Niger J Clin Pract* 2016; 19: 793-798.
20. Adejumo EN, Ogundahunsi OA, Adejumo AO, Sotunsa J, Jagun O. Prevalence of metabolic syndrome in a rural and urban community in South-West Nigeria using three different definitions. *Int J Trop Dis Health* 2017; 24: 1-9.
21. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;18: 499–502.
22. Amoah AG, Schuster DP, Gaillard T, Osei K. Insulin sensitivity and cardiovascular risk factors in hypertensive and normotensive native Ghanaians. *Diabetologia* 2003; 46: 949–955.
23. Peat J, Barton B. Medical Statistics: A Guide To Data Analysis And Critical Appraisal. BMJ Books, 2008.
24. Momin AA, Bankar MP, Bhoite GM. Determination of HOMA IR cut off value, and efficiency of lipids and lipoprotein ratios as discriminator of insulin resistance in type 2 diabetes mellitus patients. *IOSR J Pharm* 2014; 4, 9-14.
25. Li C, Ford ES, Meng YX, Mokdad AH, Reaven GM. Does the association of the triglyceride to high-density lipoprotein cholesterol ratio with fasting serum insulin differ by race/ethnicity? *Cardiovasc Diabetol* 2008; 7: 4.
26. Sumner AE, Cowie CC. Ethnic differences in the ability of triglyceride levels to identify insulin resistance. Atherosclerosis 2008; 196: 696–703.
27. Knight MG, Goedecke JH, Ricks M, Evans J, Levitt NS, Tulloch-Reid MK, et al. The TG/HDL-c ratio does not predict insulin resistance in overweight women of African descent: a study of South African, African American and West African women. *Ethn Dis* 2011; 21: 490-494.
28. Goedecke JH, Utzschneider K, Faulenbach MV, Rizzo M, Berneis K, Spinas GA, et al. Ethnic differences in serum lipoproteins and their determinants in South African women. *Metabolism* 2010; 59: 1341–1350.

ORIGINAL ARTICLE

Glycaemic control and associated platelet indices among apparently healthy caregivers in Southern Nigeria

Euphoria C Akwiwu, Margaret S Edem, Josephine O Akpotuzor, Idongesit K Isong, Anthony O Okafor and Zibril A Okhormhe

ABSTRACT

Objectives: Laboratory testing for the diagnosis and management of diabetes mellitus relies largely on the fasting blood glucose in Nigeria. Glycated haemoglobin is rarely used in Nigeria in the screening of apparently healthy subjects. The implication of this needed to be studied as it relates to platelet activation, which is altered in diabetes. This study aimed at investigating glycaemic control and platelet parameters among apparently healthy caregivers with normal fasting blood sugar levels.

Methods: This was a cross-sectional study that enrolled 134 caregivers working at the University of Calabar Teaching Hospital, Calabar, Nigeria. Fasting plasma glucose was assayed by glucose oxidase method while glycated haemoglobin was assayed by an ion exchange resin method. Platelet indices were measured using a Sysmex KX-21N Haematology Autoanalyzer.

Results: Prevalence rates for various levels of glycaemic control were obtained. Normal control and good control groups were observed to be 66.4% and 24.6% respectively. Subjects with fair control constituted 9% of the studied population, while none fell into the poor control group. Fasting blood sugar associated positively ($p = 0.011$) with mean platelet volume, while glycated haemoglobin associated negatively ($p = 0.002$) with the platelet count. There was also a positive association ($p = 0.005$) between age and glycated haemoglobin.

Conclusions: Deterioration in glycaemic control and haemostatic disturbance are present among apparently healthy adults yet to be diagnosed with diabetes.

Keywords: Diabetes mellitus, glycaemic control, platelet activation, mean platelet volume.

N Z J Med Lab Sci 2020; 74: 87-90.

INTRODUCTION

In less developed countries, workers are usually exposed to occupational problems which results in deterioration of health, safety, and wellbeing. Health challenges faced by workers arise from several factors which include poor working conditions, work overload, role in the organisation including role ambiguity and conflict, lack or inadequate healthcare services etc. These factors may increase the stress of the workers and they may adversely affect their health (1). Johnson *et al.* observed that different occupations carry different basic stressors (2). Care givers are generally exposed to stress, and the hazard involved in their work is well documented and may lead to overt health challenges (1-3). An aspect of health that has been observed among middle aged persons is that of metabolic syndrome of which Type 2 diabetes mellitus is among the commonest. In Nigeria, a systematic review and meta-analysis carried out by Uloko *et al.* [4] observed a prevalence of diabetes in Nigeria to be 5.77% and the highest prevalence of diabetes of 9.8% was observed in the South Eastern zone of Nigeria (4-6). They recommended a need for a prevention policy and for national diabetes care to be done.

Diabetes has been defined as a metabolic disorder of chronic hyperglycemia caused by insulin deficiency or insufficiency leading to changes in carbohydrate, protein and fat metabolism (7). This disease has shown an increase in prevalence in recent times (4,8). The International Diabetes Federation published a prevalence of 425 million people living with diabetes mellitus worldwide and 50% of these are undiagnosed. In Calabar, Nigeria the prevalence of undiagnosed diabetes was 7%. (7-9).

Diabetes mellitus can be diagnosed using one of the following tests: glycated haemoglobin (HbA_{1c}), fasting plasma glucose, or an oral glucose tolerance test. The use of glycated haemoglobin is well known for its usefulness in monitoring treatment compliance, but its use as a diagnostic screening test tool is controversial. (10–12). Owing to issues relating to cost, fasting plasma glucose is more readily used in the screening and diagnosis of diabetes in resource poor settings. Considering that diabetes may be under diagnosed, a necessary approach in reviewing the utility of glycated haemoglobin would require the evaluation of parameters and serve as indicators of diabetic morbidity. One such aspect involves the activation of platelets as glycation persists. It is known that there is platelet activation in diabetes which in time increases the risk of thrombosis, myocardial infarction, and stroke (13-15). However, how these morbidity indicators manifest in the undiagnosed state is yet to be fully studied.

MATERIALS AND METHODS

A cross-sectional study was carried out in the University of Calabar Teaching Hospital, Calabar, Nigeria between February and August 2019. A total of one hundred and thirty-four (134) apparently healthy care givers working there, who were aged 30 to 50, were randomly selected. Equal number of males and females participated in the study. Inclusion criteria included those who gave consent and their fasting plasma glucose was within the reference range. The fasting plasma glucose was measured for three consecutive days and the average value obtained.

Seven mL of blood was obtained and dispensed into appropriate sample containers. Platelet count, mean platelet volume (MPV), platelet distribution width (PDW), glycated haemoglobin and fasting plasma glucose assays were carried out. Fasting plasma glucose was assayed by the glucose oxidase method (Randox, UK). Glycated haemoglobin was assayed by ion exchange resin method (Spectrum, Egypt). Platelet indices were measured on a Sysmex KX-21N Haematology Autoanalyzer (Sysmex Corporation, Japan). Statistical analysis was by Pearson's correlation and ANOVA. Statistical significance was set at the p 0.05 level.

RESULTS

Prevalence rates for levels of glycemic control were set as follows; normal control <42.08 mmol/mol (<6.0 %), good control 42.08-50.82 mmol/mol (6.0-6.8 %), fair control 50.82-59.57 mmol/mol (6.8-7.65 %) and poor control >59.57 mmol/mol (>7.65 %). The normal control and good control groups were 66.4% and 24.6% respectively. Subjects with fair control constituted 9% of the studied population, while none fell into the poor control group (Table 1).

The gender distribution of the various glycaemic control groups revealed a comparable equal male to female ratio at the normal control level while a male to female ratio of 2.3:1 was observed at the good control level, while the fair control group comprised 100% females (Table 2).

Glycated haemoglobin levels were significantly different across the three groups, while platelet counts of the normal control group were significantly different compared to both the good and fair control groups. Mean platelet volumes of the good control differed significantly from both the normal and fair control groups (Table 3).

Pearson's correlation of measured parameters among the fair control group revealed the following relationships. Fasting plasma glucose was positively associated with the mean platelet volume, while glycated haemoglobin was negatively associated with the platelet count. There was also a positive association between age and glycated haemoglobin (Table 4). Apart from glycated haemoglobin, that varied significantly across the three groups, the platelet counts of normal control group varied with both the good and fair control groups. Mean platelet volume of the good control differed significantly from both the normal and fair control groups.

Table 1. Frequency distribution of glycaemic control ranges of the studied population.

Glycaemic control ranges	Frequency (n)	Percent
Normal control <42.08 mmol/mol	89	66.4%
Good control 42.08-50.82 mmol/mol	33	24.6%
Fair control 50.82-59.57 mmol/mol	12	9.0%
Poor control >59.57 mmol/mol	0	0%
Total	134	100%

Table 2. Glycaemic control ranges of the studied population by gender.

Glycaemic control ranges	Male n (%)	Female n (%)	Male:female ratio	Total n
Normal control: <42.08 mmol/mol	44 (49.4%)	45 (50.6%)	1:1	89
Good control: 42.08-50.82 mmol/mol	23 (69.7%)	10 (30.3%)	2.3:1	33
Fair control 50.82-59.57 mmol/mol	0 (0%)	12 (100%)		12
Poor control >59.57 mmol/mol	0 (0%)	0 (0%)	-	0

Table 3. Effect of glycaemic control on measured parameters.

Parameter	Normal control n=89	Good control n=33	Fair control n=12	p
Age (years)	41.61 ± 5.93	42.15 ± 3.44	45.25 ± 5.47	0.095
BMI (Kg/m^2)	28.16 ± 7.06	27.32 ± 3.12	28.06 ± 5.73	0.804
FPG (mmol/l)	4.30 ± 0.72	4.47 ± 0.80	4.68 ±0 .80	0.167
HbA1c (mmol/mol)	31.26 ± 5.71**	43.37 ± 1.43**	53.28 ± 2.14**	0.001
PLT (x10^9/l)	165.09 ± 45.76*	210.94 ± 47.94	205.17 ± 52.06	0.001
MPV (fl)	9.14 ± 0.66	8.46 ± 0 .60*	9.13 ± 0.68	0.001
PDW (%)	16.10 ± 0.96	16.15 ± 0.75	15.63 ± 0.94	0.217

Table 4. Pearson's correlation values for subjects with fair control.

Parameters	Pearson's correlation (r)	p
Fasting plasma glucose and MPV	0.702	0.011
HbA1c and platelet count	-0.805	0.002
Age and HbA1c	0.243	0.005

DISCUSSION

This study determined glycaemic control of apparently healthy adults aged 30 – 50 years whose fasting plasma glucose levels were within the reference range (3.5 - 6.7 mmol/L), and thus considered normal. Although, no subject had poor glycaemic control, a 9% prevalence was observed for fair glycaemic control while the normal control and good control groups were observed to be at a prevalence of 66.4% and 24.6%, respectively. Moreover, the group with fair glycaemic control constituted of only females whose age correlated positively with their glycated haemoglobin levels. This observation suggests a female preponderance for pre-diabetes among Nigerians. Apparently, being a female and being elderly contribute to the risk for diabetes among Nigerians.

The platelet count and mean platelet volume varied across different glycaemic control groups and interestingly, among the group with fair glycaemic control, the fasting blood sugar correlated positively with the mean platelet volume, while glycated haemoglobin had a negative relationship with the platelet count. This finding suggests that challenged glycaemic control associates with complications of diabetes with regards to platelet indices even before the onset of diabetes. There is platelet activation as glycaemic control becomes less effective. (15-17). The implications of this among persons thought to be healthy is quite worrisome. Persistent hyperglycaemia, as well as challenged glycaemic control, leads to glycation of proteins to the detriment of physiological function (18 19). The loss of functional integrity eventually manifests as complications that contribute to morbidity and mortality. Endothelial dysfunction and direct glycation of platelets are thought to give rise to platelet activation which in turn promotes haemostatic disturbance in diabetes (20,21). Platelet activation is reflected in increased mean platelet volume as it is indicative of increased synthesis.

In the presence of depleting platelet count, higher mean platelet volume reflects a compensatory response by the body. It is therefore plausible that haemostatic complications could already be present even before the diagnosis of diabetes is made (22-25). It might be necessary to consider platelet parameters early in the management of diabetes and in population screening for diabetes. Considering that haemostatic complications could already be present even before the diagnosis of diabetes is made using fasting plasma glucose, it would be appropriate to routinely incorporate glycated haemoglobin in the screening and diagnosis of diabetes.

In conclusion, deterioration in glycaemic control and haemostatic disturbance are present among apparently healthy adults yet to be diagnosed with diabetes. Therefore, dependence on the fasting blood sugar alone for screening of populations is inadequate. Glycated haemoglobin should be incorporated in the routine screening and management of diabetes.

AUTHOR INFORMATION

Euphoria C Akwiwu, BMLS MSc PhD, Lecturer[1]
Margaret S Edem, BMLS MSc, Assistant Director Medical Laboratory[2]
Josephine O Akpotuzor, BSc MSc PhD, Professor[1]
Idongesit K Isong, BSc MSc PhD, Lecturer[1]
Anthony O Okafor, AIMLS BMLS MSc PhD, Chief Medical Laboratory Scientist[3]
Zibril A Okhormhe, AIMLS, Chief Medical Laboratory Scientist[1]

[1]Department of Medical Laboratory Science, University of Calabar, Calabar, Nigeria
[2]Department of Haematology, University of Calabar Teaching Hospital, Calabar, Nigeria
[3]Department of Chemical Pathology, University of Calabar Teaching Hospital, Calabar, Nigeria

Corresponding author: Dr. Idongesit Isong.
Email: ikpisong@gmail.com

REFERENCES

1. Maulik PK. Workplace stress: A neglected aspect of mental health wellbeing. *Indian J Med Res* 2017; 146: 441–444.
2. Johnson, S, Cooper C, Cartwright S, et al. The experience of work-related stress across occupations. *J Manag Psychol* 2005; 20: 178-187.
3. Mezuk B, Eaton WW, Albrecht S, Golden SH. Depression and type 2 diabetes over the lifespan: A meta-analysis. *Diabetes Care* 2008; 31: 2383–2390.
4. Uloko AE, Musa BM, Ramalan MA, et al. Prevalence and risk factors for diabetes mellitus in Nigeria: a systematic review and meta-analysis. *Diabetes Ther* 2018; 9: 1307–1316.
5. Uloko AE, Ofoegbu EN, Chinenye S, et al. Profile of Nigerians with diabetes mellitus—Diabcare Nigeria Study Group (2008): results of a multicenter study. *Indian J Endocrinol Metab* 2012; 16: 558–564.
6. Nyenwe EA, Odia OJ, Ihekwaba AE, et al. Type 2 diabetes in adult Nigerians: a study of its prevalence and risk factors in Port Harcourt, Nigeria. *Diabetes Res Clin Pract* 2003; 62: 177–185.
7. World Health Organization. Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications, Part 1. WHO, Geneva, 1999.
8. International Diabetes Federation. Diabetes Atlas. 8th ed. International Diabetes Federation Brussels, 2017.
9. Enang OE, Otu AA, Essien OE, et al. Prevalence of dysglycemia in Calabar: a cross-sectional observational study among residents of Calabar, Nigeria. *BMJ Open Diabetes Res Care* 2014; 2: e000032.
10. Davidson MB, Schriger DL, Peters AL, Lorber B. Relationship between fasting plasma glucose and glycosylated hemoglobin: potential for false-positive diagnoses of type 2 diabetes using new diagnostic criteria. *JAMA* 1999; 281:1203–*1210.*
11. McCance DR, Hanson RL, Charles MA, et al. Comparison of tests for glycated haemoglobin and fasting and two hour plasma glucose concentrations as diagnostic methods for diabetes. *BMJ 1994; 308: 1323-1328.*
12. Peters AL, Davidson MB, Schriger DL, Hasselblad V. A clinical approach for the diagnosis of diabetes mellitus: an analysis using glycosylated hemoglobin levels. Meta-analysis research group on the diagnosis of diabetes using glycated hemoglobin levels. *JAMA* 1996; 276:1246–*1252.*
13. Hasegawa Y, Suehiro A, Higasa S, et al. Enhancing effect of advanced glycation end products on serotonin-induced platelet aggregation in patients with diabetes mellitus. *Thromb Res* 2002; 107: 319-323.
14. Han Y, Liu Y, Mi Q, et al. Pyridoxine improves platelet nitric oxide synthase dysfunction induced by advanced glycation end products in vitro. *Int J Vitam Nutr Res* 2010; 80: 168-177.
15. Jindal S, Gupta S, Gupta R, et al. Platelet indices in diabetes mellitus: indicators of diabetic microvascular complications. *Hematology* 2011; 16: 86-89.
16. Shah B, Sha D, Xie D, et al. The relationship between diabetes, metabolic syndrome, and platelet activity as measured by mean platelet volume. The National Health and Nutrition Examination Survey, 1999-2004. *Diabetes Care* 2012; 35: 1074-1078.
17. Chen, X, Fang L, Lin H, et al. The relationship between type 2 diabetes and platelet indicators. *Iran J Public Health* 2017; 46: 1211-1216.
18. Sell DR, Monnier VM. Molecular basis of arterial stiffening: role of glycation – a mini-review. *Gerontology* 2012; 58: 227-237.

19. Kaur R, Kaur M, Singh, J. Endothelial dysfunction and platelet hyperactivity in type 2 diabetes mellitus: molecular insights and therapeutic strategies. *Cardiovasc Diabetol* 2018; 17: 121.
20. Sobol AB, Watala C. The role of platelets in diabetes-related vascular complications. *Diabetes Res Clin Pract* 2000; 50: 1-16.
21. Winocour PD. The role of platelets in the pathogenesis of diabetic vascular disease. In: Complications of Diabetes Mellitus. Molecular and Cellular Biology of Diabetes Mellitus, Vol. III. B Draznin, S Melmed, D LeRoith. (eds) Alan R Liss Inc., New York, 1989, pp 37-47.
22. Grant PJ. Diabetes mellitus as a prothrombotic condition. *J Intern Med* 2007; 262: 157-172.
23. Zuberi B, Akhtar N, Afsar S. Comparison of mean platelet volume in patients with diabetes mellitus, impaired fasting glucose and non-diabetic subjects. *Singapore Med J* 2008; 49: 114-116.
24. Schneider DJ. Factors contributing to increased platelet reactivity in people with diabetes. *Diabetes Care* 2009; 32: 525-527.
25. Vinik AI, Erbas T, Park TS, et al. Platelet dysfunction in type 2 diabetes. *Diabetes Care* 2001; 24: 1476-1485

ORIGINAL ARTICLE

Crisis frequency and associated changes in platelet parameters among steady state sickle cell subjects

Euphoria C Akwiwu, Eme E. Onukak, Idongesit K Isong, Josephine O Akpotuzor, Iya Eze Bassey and Anthony O Okafor

ABSTRACT

Objectives: The management of sickle cell anaemia is geared towards resolving sickling crisis as well as possible prevention of crisis for those in steady state. Crisis frequencies vary from person to person and among populations. Its impact, however, extends to morbidity-associated consequences even in steady state. This study investigated crisis frequency of sickle cell anaemia subjects in relation to their platelet parameters.

Methods: Subjects comprised 45 male and female sickle cell anaemia patients attending clinic at University of Calabar Teaching Hospital Calabar, Nigeria and equal number of age and sex-matched control subjects with homozygous adult haemoglobin (HbAA). Platelet parameters were analysed by automation using a SMART-1 Hematology Analyzer. Foetal haemoglobin estimation was performed using a modified Betke's method.

Results: The platelet count and plateletcrit of sickle cell anaemia patients were significantly higher compared to control subjects. The mean platelet volume was significantly higher among subjects from 30 years of age compared to younger age groups. Initial increase in crisis frequency brought about depletion in the platelet count and plateletcrit. The plateletcrit stabilised as the mean platelet volume picked up at 4-7 crisis frequency within the preceding one year.

Conclusion: Increase in crisis frequency and longer years of living with sickle cell anaemia resulted in higher platelet involvement.

Keywords: sickle cell anaemia, crisis frequency, HbAA, plateletcrit, platelet volume.

N Z J Med Lab Sci 2020; 74: 91-94.

INTRODUCTION

Distribution of haemoglobin types in Nigeria favours the homozygous adult haemoglobin (HbAA) as the prevailing phenotype. However, the sickling gene remains present in the form of HbAS and HbSS phenotypes, with the later manifesting as sickle cell anaemia (1-3). Sickle cell anaemia remains a health challenge in Nigeria despite its discovery over a century ago. Efforts to see to the possible eradication of the condition through genetic counselling for prospective couples with the recessive gene have been encouraged through sustained enlightenment campaigns (4,5). Notwithstanding, children are still born with this condition, particularly in rural Nigeria, due to inadequate knowledge on the part of uninformed couples (6). Sickle cell anaemia continues to contribute to deaths in under five-year olds in Africa even though the condition is avoidable (7,8). Fortunately, access to adequate healthcare greatly improves the survival of affected individuals into adult life. Expert counselling, provision of prompt access to needed care as well as commitment and dedication of medical staff among other factors contributes significantly to improved survival and quality of life (9).

In sickle cell anaemia, the switch from foetal haemoglobin (HbF) to HbS is delayed. As a result, stable levels of HbF are reached later than usual. Most adults affected with sickle cell anaemia have increased levels of HbF which is thought to be of benefit by ameliorating the sickling process, reducing crisis frequency and impacting positively on quality of life. The extent of increase in HbF is, however, highly variable from one population to the other (10,11).

The clinical features of sickle cell disease are defined by chronic anaemia, haemolysis, sepsis, and recurrent acute vaso-occlusive crisis (12). Increase in oxidative stress, the inflammatory response, haematological changes and endothelial activation after a period of hypoxia/reoxygenation have all been demonstrated in sickling models (13-16). A significant aspect of the pathogenesis of sickle cell disease involves inflammation, accompanied by heterocellular leukocyte-platelet-erythrocyte-endothelial adhesive events that trigger vaso-occlusive episodes, acute organ ischemia, and reperfusion injury (17). It is therefore plausible to hypothesize on a link between crisis frequency and morbidity indicators, such as a triggered coagulation response. The underlying mechanism for higher platelet count in sickle cell anaemia and how it relates to severity of the condition remains largely unclear.

METHODS

This study was carried out among consenting adult steady-state sickle cell anaemia subjects attending clinic at University of Calabar Teaching Hospital, Calabar, Nigeria. The study subjects included 18 male and 27 female sickle cell anaemia patients with equal number of age and sex-matched control subjects with HbAA. Ethical approval was obtained from The Health and Research Ethics Committee of University of Calabar Teaching Hospital, while informed consent was obtained from each participant. Information on crisis frequency for the preceding one year was retrieved from patients' records.

A venous blood sample was aseptically collected from each participant into labelled ethylenediaminetetraacetic acid bottles for analysis of platelet parameters and foetal haemoglobin estimation. The platelet parameters were analysed by automation using SMART-1 Hematology Analyzer (Kinghawk Technology Co., Ltd, China). Foetal haemoglobin estimation was performed using the modified Betke method (18).

Statistical analysis of data (student t-test and one-way analysis of variance) was done using SPSS 22.0. Statistical significance was set at the p 0.05 level. Data are presented as numbers with percentages and as means with standard deviations.

RESULTS

A total of 45 male and female subjects between the ages of 18 - 40 were enrolled in the study. The age group with the highest number of participants was 20-29 years constituting 48.9% of the subjects. The least number of participants were those above 30 years of age constituting 17.8% of the subjects. Females constituted 60% of the subjects, while males constituted 40% (Table 1).

Platelet count and plateletcrits of sickle cell anaemia patients were significantly higher compared to control subjects (Table 2). Mean platelet volumes were significantly higher among subjects ≥30 years of age compared to those ≤19 years of age (Table 3). Platelet counts and plateletcrits were significantly lower among subjects that had a crisis 1-3 times within the preceding one year compared to the other groups, while mean platelet volumes were significantly higher among subjects that had a crisis 4-7 times within the preceding one year compared to other categories under crisis frequency categories (Table 4).

Table 1. Demographic parameters of sickle cell anaemia patients and controls.

Parameters	Patients Number (%)	Controls Number (%)
Age		
<19 years	15 (33.3)	14 (31.1)
20-29 years	22 (48.9)	23 (51.1)
≥30years	8 (17.8)	8 (17.8)
Females	27 (60)	27 (60)
Males	18 (40)	18 (40)
Crisis frequency in the preceding year		
No crisis	10 (22.2)	-
1-3 times	27 (60)	-
4-7 times	8 (17.8)	-
Total	45 (100)	-
Routine check		
Once every month	10 (22.2)	-
Twice every month	4 (8.9)	-
Once in two months	10 (22.2)	-
Occasionally	21 (46.7)	-

Table 2. Platelet parameters of sickle cell anaemia patients and controls.

Parameters	Patients (n=45)	Controls (n=45)	p
PLT ($\times 10^9$/l)	352.73 ± 109.49	195.00 ± 56.98	0.001
PDW (%)	15.92 ± 1.04	15.50 ± 1.33	0.092
MPV (fl)	8.76 ± 0.64	8.74 ± 0.69	0.875
PCT (%)	0.30 ± 0.09	0.16 ± 0.05	0.001
P-LCR (%)	17.30 ± 5.05	17.65 ± 5.62	0.758
Hb F (%)	8.82 ± 2.27	2.20 ± 1.29	0.001

Results are mean ± standard deviation. PLT=Platelet, PDW=Platelet distribution width, MPV=Mean platelet volume, PCT=Plateletcrit, P-LCR= Platelet large cell ratio, Hb F=Haemoglobin F

Table 3. Age groups and platelet parameters of sickle cell anaemia patients.

Parameters	≤19 years (n=15)	20-29 years (n=22)	≥30 years (n=8)	p
PLT (×10^9/l)	338.80 ± 109.85	368.00 ± 113.14	336.88 ± 106.68	0.668
PDW (%)	15.23 ± 1.30	15.71 ± 1.22	15.40 ± 1.71	0.560
MPV (fl)	8.47 ± 0.40	8.72 ± 0.63	9.33 ± 0.98 *	0.014
PCT (%)	0.29 ± 0.08	0.31 ± 0.10	0.30 ± 0.12	0.758
P-LCR (%)	17.19 ± 5.30	16.74 ± 4.90	21.05 ± 7.36	0.166
Hb F (%)	8.79 ± 2.33	9.01 ± 2.46	8.34 ± 1.78	0.779

Results are mean ± standard deviation. *Statistically different from ≤19 years group.
PLT=platelets; PDW=platelet distribution width; MPV=mean platelet volume; PCT=plateletcrit; P-LCR= platelet large cell ratio; Hb F=haemoglobin F.

Table 4. Platelet parameters of sickle cell anaemia patients with different crisis frequencies.

Parameters	No crisis (n=10)	1-3 times (n=27)	4-7 times (n=8)	p
PLT (×10^9/l)	408.70 ± 120.83	315.59±102.51*	408.13 ± 65.15	0.017
PDW (%)	15.77 ± 1.23	15.41 ± 1.34	15.45 ± 1.51	0.766
MPV (fl)	8.46 ± 0.68	8.70 ± 0.62	9.24 ± 0.78**	0.050
PCT (%)	0.34 ± 0.08	0.26 ± 0.09*	0.37 ± 0.04	0.002
P-LCR (%)	15.41 ± 4.97	18.03 ± 5.15	19.19 ± 7.61	0.322
Hb F (%)	8.59 ± 2.03	9.02 ± 2.28	8.44 ± 2.74	0.777

Results are mean ± standard deviation. *Statistically different from no crisis group. **Statistically different from no crisis and 1-3 times groups.
PLT=platelets; PDW=platelet distribution width; MPV=mean platelet volume; PCT=plateletcrit; P-LCR= platelet large cell ratio; Hb F=haemoglobin F.

DISCUSSION

Routine medical checkups are a critical factor to the overall well-being of people living with sickle cell anaemia. The present study observed that 46.7% of the participants showed up for routine medical checkups occasionally. The most recurrent reason for irregular medical checkup visits among the participants was financial constraint. Sickle cell anaemia is still a healthcare challenge in Nigeria. The ability for affected persons to access conventional healthcare is hampered by lack of a universal national health insurance package. Not all the participants could afford regular medical checks and crisis frequency varied from none to seven times within the preceding one year. Unfortunately, significant changes in platelet parameters accompanied an increase in crisis frequency. Platelet counts and plateletcrits witnessed an initial depletion among subjects that had a crisis 1-3 times within the preceding one year but appeared to pick up with increased crisis frequency. This line of thought is further supported by the finding of higher mean platelet volumes among subjects that had a crisis 4-7 times within the preceding one year, a reflection of increased synthesis as crisis increases. Among the markers of platelet activation, the mean platelet volume is thought to be indicative of coagulation disturbance as it suggests increased platelet synthesis (19-21).

The involvements of the formed elements of blood in mediating vaso-occlusive crisis in sickle cell anaemia has necessitated the investigation of changes in peripheral blood cells. Previous studies have shown higher platelet counts in sickle cell anaemia patients, a finding that has been attributed to asplenia (22). The observation of increased mean platelet volume with both longer years of living with the condition as well as with increased crisis frequency, however, suggests that a higher platelet count in sickle cell anaemia condition may also be arising directly from increased synthesis. Moreover, HbF, which is thought to ameliorate sickling, remains an important aspect in body mechanisms for coping with the condition. The compensatory increase in HbF levels vary considerably by populations (10,11). The HbF mean value was about four times higher among the sickle cell anaemia patients compared to the controls, but did not vary significantly by age. The HbF values in this Nigerian population have no apparent impact on crisis frequency, however, HbF was also found to have significant negative correlation with the platelet large cell ratio, indicating that individually, lesser amelioration of sickling coexisted with increasing platelet synthesis. This has implications for increased risk of haemostatic dysfunction in association with sickle cell anaemia, particularly when crisis frequency is left uncontrolled.

Limitations of this study include the use of a manual staining and counting method prone to some degree of variability for estimation of HbF. Additionally, disproportionate sample sizes fell into some of the categories with regards to age and crisis frequency. It was mainly because not many sickle cell patients survive through adult life, especially in resource-poor settings. In conclusion, the present study observed that increased crisis frequency and longer years of living with sickle cell anaemia resulted in higher platelet involvement.

AUTHOR INFORMATION

Euphoria C Akwiwu, BMLS MSc PhD, Lecturer[1]
Eme E Onukak, BMLS MSc, Student/Researcher[1]
Idongesit K Isong, BSc MSc PhD, Lecturer[1]
Josephine O Akpotuzor, BSc MSc PhD, Professor[1]
Iya Eze Bassey, BSc MSc PhD, Lecturer[1]
Anthony O Okafor, AIMLS BMLS MSc PhD, Chief Medical Laboratory Scientist[2]

[1]Department of Medical Laboratory Science, University of Calabar, Calabar, Nigeria
[2] Department of Chemical Pathology, University of Calabar Teaching Hospital, Calabar, Nigeria

Corresponding Author: Euphoria Akwiwu.
Email: ecakwiwu@gmail.com

REFERENCES

1. Akhigbe R.E, Ige SF, Afolabi AO, et al. Prevalence of haemoglobin variants, ABO and Rhesus blood groups in Ladoke Akintola University of Technology, Ogbomoso, Nigeria. *Trends Med Res* 2009; 4: 24-29.
2. Umoh AV, Abah GM, Ekanem TI, Essien EM. Haemoglobin genotypes: a prevalence study and implications for reproductive health in Uyo, Nigeria. *Niger J Med* 2010; 19: 36-41.
3. Akwiwu EC, Usanga EA, Akpotuzor JO. Comparative study of haemoglobin types in coastal (Yenogoa) and hinterland (Owerri) communities in South-Eastern Nigeria. *J Med Lab Sci* 2011; 20: 16-20.
4. Odunvbun ME, Okolo AA, Rahimy CM. Newborn screening for sickle cell disease in a Nigerian hospital. *Public Health* 2008; 122: 1111-1116.
5. Aneke JC, Okocha CE. Sickle cell disease genetic counselling and testing: a review. *Arch Med Health Sci* 2016; 4: 50-57.
6. Akwiwu EC, Akpotuzor JO, Okafor AO. Knowledge of haemoglobin types at union among couples and impact on offspring; a cross-sectional study in Southern Nigeria. *Asian Hematol Res J* 2019; 2: 1-6.
7. Grosse SD, Odame I, Atrash HK, et al. Sickle cell disease in Africa: a neglected cause of early childhood mortality. *Am J Prev Med* 2011; 41(6 Suppl 4): S398–S405.
8. McGann PT. Sickle cell anemia: an underappreciated and unaddressed contributor to global childhood mortality. *J Pediatr* 2014; 165: 18–22.
9. Akinyanju OO, Otaigbe, AI, Ibidapo, MO. Outcome of holistic care in Nigerian patients with sickle cell anaemia. *Clin Lab Haematol* 2005; 27: 195-199.
10. Sankaran VG, Xu J, Orkin SH. Advances in the understanding of haemoglobin switching. *Br J Haematol* 2010; 149: 181-194.
11. Solovieff N, Milton JN, Hartley SW, et al. Fetal haemoglobin in sickle cell anemia: genome-wide association studies suggest a regulatory region in the 5' olfactory receptor gene cluster. *Blood* 2010; 115: 1815-1822.
12. Chakravorty S, Williams TN. Sickle cell disease: a neglected chronic disease of increasing global health importance. *Arch Dis Child* 2015; 100: 48-53.
13. Kaul DK, Hebbel RP. Hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice. *J Clin Invest* 2000; 106: 411–420.
14. Guzik TJ, Korbut R, Adamek-Guzik T. Nitric oxide and superoxide in inflammation and immune regulation. *J Physiol Pharmacol* 2003; 54: 469–487.
15. Omoti CE. Haematological values in sickle cell anaemia in steady state and during vaso-occlusive crisis in Benin City, Nigeria. *Ann Afr Med* 2005; 4: 62-67.
16. De Franceschi L, Cappellini MD, Olivieri O. Thrombosis and sickle cell disease. *Semin Thromb Hemost* 2011; 37: 226-236.
17. Kato GJ, Steinberg MH, Gladwin MT. Intravascular hemolysis and the pathophysiology of sickle cell disease. *J Clin Invest* 2017; 127: 750-760.
18. Betke K, Marti HR, Schlicht I. Estimation of small percentage of foetal haemoglobin. *Nature* 1959; 184 (Suppl 24): 1877-1878.
19. Ntolios P, Papanas E, Nena E, et al. Mean platelet volume as a surrogate marker for platelet activation in patients with idiopathic pulmonary fibrosis. *Clin Appl Thromb Hemost* 2016; 22: 346-350.
20. Chu SG, Becker RC, Berger PB, et al. Mean platelet volume as a predictor of cardiovascular risk: a systematic review and meta-analysis. *J Thromb Haemost* 2010; 8: 148-156.
21. Celik T, Unal S, Ekinci O, et al. Mean Platelet Volume can predict cerebrovascular events in patients with sickle cell anemia. *Pak J Med Sci* 2015; 31: 203-208.
22. Okpala I. Steady-state platelet counts and complications of sickle cell disease. *Hematol J* 2002; 3: 214-215.

ORIGINAL ARTICLE

The diagnostic value of neuron-specific enolase in patients with mild head injury requiring cranial CT scan

Javad Mozafari, Hassan Barzegari, Hassan Motamed, Mohammad Ghasem Hanafi and Ramin Faghihi

ABSTRACT

Objectives: The purpose of this study was to investigate the relationship between changes in serum neuron-specific enolase level and changes in CT scan findings in patients with mild head injury associated with intracranial subdural haematoma.

Methods: An observational cross-sectional study was conducted on adult patients with mild head trauma associated with acute subdural haematoma referred to the Emergency Department of Ahvaz Golestan Hospital between 2017-2018. Of all patients who met the inclusion criteria, a venous serum sample was first taken during the first three hours of the accident and just before performing the cranial CT scan, and then the second sample was taken just before performing the CT scan again.

Results: A total of 40 patients with mean age of 35 ± 2.1 years were entered the study. 92.5% of the patients were male. The results of the receiver operating characteristics (ROC) curve showed that the sensitivity, specificity, and accuracy of the primary enolase with a cut-off point of 10.74 in the diagnosis of intracranial haemorrhage were 44%, 86%, and 58%, respectively. Also, the results of the ROC curve showed that, the sensitivity, specificity, and accuracy of the secondary enolase in the diagnosis of intracranial haemorrhage was 94%, 64% and 81%, respectively, at a cut-off point of 13.10.

Conclusion: The results of this study indicated that increased intracranial haemorrhage volume in secondary CT is associated with an increase in the level of primary enolase serum concentration.

Keywords: head trauma, neuron-specific enolase, cranial CT scan.

N Z J Med Lab Sci 2020; 74: 95-97.

INTRODUCTION

Traumatic head injury is one of the leading causes of death and disability in neurosurgery and neurology care. Despite significant advances in brain monitoring, it is still difficult to estimate primary and secondary brain injury by means of the Glasgow Coma Scale or primary CT scans (1). Worldwide, more than 11 million people are killed or hospitalised each year due to traumatic brain injuries. About 71% of deaths caused by these injuries occur in low- or middle-income countries. These lesions occur mostly at a young age. Most of the disabilities and costs associated with traumatic brain injury are hidden, such that affected people may have no clinical manifestation of this injury (2). Many of these patients will experience permanent or long-term disabilities. The social and economic burden of these injuries is significant (3).

Subdural haemorrhage is the most common intracranial focal lesion, which is seen in 24% of patients with severe closed head injury (4). The site of this bleeding is located between the dura and the brain. It is usually the result of the rupture of bridging veins between the cortex and the discharging sinuses and often found in the frontoparietal region. Mortality in this type of bleeding has been reported to be between 50 and 60% (5). With the rapid increase in motor vehicles, the incidence of these injuries is projected to increase in low- and middle-income countries (6,7). The mortality rate varies according to the severity of the injury, but it is high in severe injuries and the elderly (5). Head CT scans are the main diagnostic imaging tool in acute cases that can be used to determine prognosis and clinical outcomes (8). The comparison between abnormal CT scan findings rate, which is 6% in unselected head injury patients, and estimates of head injury incidence rate suggest that CT scan is insufficiently sensitive for this purpose (9).

Enolase is a dimeric cytoplasmic enzyme that acts on the glycolytic pathway and is composed of three distinct subgroups called, α, β, and y (10). The gamma part is common in neurons and neuroendocrine cells in the forms of αy and yy, which is why it is called neuron-specific enolase. Neuron-specific enolase is released both in serum and in cerebrospinal fluid after injury to the central nervous system. Therefore, it seems that it could act as a biomarker in the degradation processes of central nervous system (11). The aim of this study was to investigate the relationship between changes in serum neuron-specific enolase level and changes in CT scan findings in patients with mild head injury associated with intracranial subdural haematoma.

METHODS

Study design

An observational cross-sectional study was conducted on adult patients with mild head trauma associated with acute subdural haematoma referred to the Emergency Department of Ahvaz Golestan Hospital, Ahvaz, Iran between 2017-2018. The study was carried out in accordance with the Declaration of Helsinki, a statement of ethical principles for medical research involving human subjects and after approval from the Ethics Committee of Jundishapur University of Medical Sciences, Ahvaz, Iran.

Participants

All patients older than 18 years with mild head trauma associated with acute subdural haematoma who were not candidates for emergency neurosurgery surgery and had Glasgow Coma Scale scores 7-13 were entered the study. Exclusion criteria included unwillingness to participate in the study, any fractures or serious injuries in other parts of the body, a history of neurosurgery, pregnancy, previous history of alcohol or drug abuse, previous neurological disease such as seizures, and epilepsy.

Tests

From all patients who met the inclusion criteria, after obtaining initial information, initial examinations and primary stabilisation,

a venous blood sample was first taken by the ward nurse during the first three hours of the accident just before performing the cranial CT scan, and then the second sample was taken just before performing the CT scan again (as secondary CT scan, 7 hours after performing the primary CT scan). The venous serum samples were stored at -20 ° C until analysis and the serum concentration of neuron-specific enolase was determined by enzyme-linked immunosorbent assay (ELISA) (CanAg Humn NSE ELI, Switzerland) in a single batch.. Primary and secondary CT scans of all patients were independently interpreted in terms of the presence, type, and progression of intracerebral lesions by the same radiologist who was unaware of the results of the serum neuron-specific enolase levels.

Data analysis

Descriptive statistical methods were used to determine the mean and standard deviation. The ROC curve was used to determine the optimal cut-off point for the serum level of neuron-specific enolase and to determine the sensitivity and specificity. An independent two-sample t-test or Mann-Whitney U test were used to compare quantitative variables between groups under study in terms of data normality. Data were analysed using IBM SPSS 22 software (SPSS Inc., IBM, Chicago, IL, USA). Statistical significance was set at the p 0.05 level.

RESULTS

A total of 40 patients (mean age 35 ± 2.1 years) were entered the study, 37 of whom were male. The majority of patients were in the age range of 20-30 and 40-50 years. Eleven of the study participants had a GCS score of 9 or 12 at the time of referral while 18 patients had haematoma in the temporal region (Table 1). The mean volumes of cerebral haemorrhage in the primary and secondary CT scans were 15893.54- and 35190.53- milli cubic centimetre (ml cc), respectively. Mean values of the primary and secondary enolase were 9.26 ng/dl and 23.83 ng/dl respectively (Table 2).

There was no statistically significant relationships between the volume of cerebral haemorrhage in the primary CT scan and changes in cerebral haemorrhage and primary enolase changes. However, there was a significant and direct relationship between the volume of cerebral haemorrhage in the secondary CT scan and the level of changes in serum primary enolase. As the volume of cerebral haemorrhage in the secondary CT scan increased, the concentration of primary enolase increased and vice versa (Table 3).

There was also no significant relationship between the mean secondary enolase level and its changes and the average GCS score of patients. However, there was a significant relationship between the mean of the serum primary enolase concentration and GCS score of patients. The average GCS score increased as the primary enolase concentration increased, and vice versa (Table 3). The ROC curve showed that the sensitivity, specificity and accuracy of the primary enolase with a cut-off point of 10.74 in the diagnosis of intracranial haemorrhage were 44, 86 and 58%, respectively. Also, the ROC curve indicated that the sensitivity, specificity and accuracy of the secondary enolase in the diagnosis of intracranial haemorrhage were 94, 64 and 81% at a cut-off point of 13.10.

DISCUSSION

Traumatic brain injury is caused by external pressure to the brain that causes temporary or permanent neurological dysfunction. In recent years, biochemical markers have been used to detect a variety of diseases. Creatinine, for example, is used for renal failure, troponin for myocardial infarction, and lipase for pancreatitis (10). The results of our study showed that there was no significant relationship between the mean serum primary enolase concentration and the mean intracranial haemorrhage volume in the primary CT scan. In other words, the serum concentration of the primary enolase is not a suitable factor for determining intracranial haemorrhage in these patients. However, there was a significant relationship between the mean serum primary enolase concentration and the mean intracranial haemorrhage volume in the secondary CT scan.

Meric *et al*. investigated the relationship between serum enolase levels and the Glasgow Coma Score in trauma patients in the emergency department (12). Their prospective study was performed on 80 trauma patients who were divided into four groups. The first group included patients with the whole-body lesions but no head injury, the second group had minor trauma, the third group had moderate head injury, and the fourth group had severe injury. The results showed that the cutoff point for neuron-specific enolase was 20.52 ng/dl. There was a statistically different in the mean enolase level between the first group (general trauma) and the third (moderate trauma) and the fourth groups (severe trauma). Their results also showed that there was a significant inverse relationship between the serum enolase levels and Glasgow Coma Scores in the third and fourth groups. As noted, with increasing serum enolase level, the Glasgow Coma Score decreases. Finally, the ROC analysis results of that study showed that serum enolase had a 87% sensitivity, 82% specificity and 93% accuracy in predicting neurological outcomes (12).

In another study of 29 patients with subarachnoid haemorrhage due to cerebral aneurysm rupture, results showed that serum enolase levels were significantly higher in patients with critical neurological condition compared with patients with good condition. Overall, the results of that study suggest that patients with a good outcome have lower levels of serum enolase. Also, serum enolase levels increased with the development of ischemic neurological deficits and this increase was observed up to three weeks after subarachnoid haemorrhage (11).

In another study that aimed to determining serum enolase as a biomarker in predicting short-term outcomes in children with traumatic brain injury and performed on 90 patients referred to the emergency department, results showed that there was a statistically difference in serum enolase levels in patients with critical condition, compared to patients with the desired condition. In that study ROC analysis results for serum enolase based on adverse and favorable outcomes showed 83% accuracy. Also, the cut-off point for the enolase level was 21.2 ng/dl with 86% sensitivity and 74% specificity. Finally, the authors suggested that enolase levels can be a predictor of short-term outcomes in children with traumatic brain injury (13).

In a systematic review and meta-analysis by Cheng et al. aimed to determine the predictive value of serum enolase in traumatic brain injuries, the results showed that among the 16 eligible articles for the study, six studies comparing the serum enolase levels in patients who survived and died of traumatic brain injury showed that the serum enolase concentration was significantly associated with mortality. In addition, a meta-analysis of patients with adverse Glasgow Coma Score outcomes showed higher serum enolase concentrations than those with favorable results. The ROC analysis results showed that the sensitivity and specificity of serum enolase for mortality were 78% and 50% and for adverse neurological prognosis were 72% and 66%, respectively (14). The results of our study indicated that serum levels of enolase increase with time since the accident happened. This may be due to the skeletal structure of the skull, which has limited space and increases blood pressure in the space, putting more pressure on the surrounding neurons and damaging them and releasing substances in them, especially enolase protein.

In conclusion, our study showed that an increase in intracranial haemorrhage in the secondary CT scan is associated with an increase in the serum primary enolase concentration, and primary enolase can be used to reduce the need for performing CT scan in these patients, reduce excess radiation to the patient, and accelerate the management of patients with mild head trauma as well. Our results also showed that secondary enolase with high accuracy and sensitivity has high predictive power to diagnose patients with cerebral haematoma expansion. Limitations of our study were the disproportionate number of patients and the relatively small sample size.

Table 1. Frequency and mean distribution of demographic and clinical variables of patients according to normal and abnormal groups.

Variables		
Gender, N (%)	**Female**	3 (7.5)
	Male	37 (92.5)
Age, Years, Mean ± SD		35 ± 2.1
Age, Years, N (%)	20 – 30	16 (40)
	30 - 40	9 (22.5)
	40 - 50	15 (37.5)
Glasgow Coma Scale, N (%)	9	11 (27.5)
	10	8 (20)
	11	10 (25)
	12	11 (27.5)
Haematoma location, N (%)	Temporal	18 (45)
	Frontal	9 (22.5)
	Partial	13 (32.5)

Table 2. Mean volume of cerebral haemorrhage in the primary and secondary CT scans and enolase levels.

Variables	Mean ± SD
Mean volume of cerebral haemorrhage in the primary CT scans, milli cubic centimetre (ml cc)	15893.54 ± 2564.37
Mean volume of cerebral haemorrhage in the secondary CT scans, milli cubic centimetre (ml cc)	35190.53 ± 3034.49
Primary enolase, ng/dl	9.26 ± 8.72
Secondary enolase, ng/dl	23.83 ± 22.09

Table 3. The correlation coefficient between variables.

Variables		The correlation coefficient	P-Value
Mean volume of cerebral haemorrhage in the primary CT scans, milli cubic centimetre (ml cc)	Primary enolase	0.091	0.575
	Secondary enolase	-0.053	0.745
Mean volume of cerebral haemorrhage in the secondary CT scans, milli cubic centimetre (ml cc)	Primary enolase	0.334	0.035 *
	Secondary enolase	0.279	0.081
Primary enolase - GCS		0.346	0.029 *
Secondary enolase - GCS		0.193	0.232

AUTHOR INFORMATION

Javad Mozafari, MD, Assistant Professor
Hassan Barzegari, MD, Assistant Professor
Hassan Motamed, MD, Assistant Professor
Mohammad Ghasem Hanafi, MD, Associate Professor
Ramin Faghihi, MD, Emergency Medicine Specialist

Department of Emergency Medicine, Golestan Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Corresponding author: Dr. Hassan Barzegari,
Email: h_b1979@yahoo.com

REFERENCES

1. Guzel A, Er U, Tatli M, et al. Serum neuron-specific enolase as a predictor of short-term outcome and its correlation with Glasgow Coma Scale in traumatic brain injury. *Neurosurg Rev* 2008; 31: 439-444; discussion: 444-445.
2. Khan F, Baguley IJ, Cameron ID. 4: Rehabilitation after traumatic brain injury. *Med J Aust* 2003; 178: 290-295.
3. Zehtabchi S, Abdel Baki SG, Falzon L, Nishijima DK. Tranexamic acid for traumatic brain injury: a systematic review and meta-analysis. *Am J Emerg Med* 2014; 32: 1503-1509.
4. Thurman DJ. The epidemiology of traumatic brain injury in children and youths: a review of research since 1990. *J Child Neurol* 2016; 31: 20-27.
5. Bruns J Jr, Hauser WA. The epidemiology of traumatic brain injury: a review. *Epilepsia* 2003; 44(s10): 2-10.
6. Peden M, Scurfield R, Sleet D, et al. World report on road traffic injury prevention. World Health Organization, Geneva; 2004.
7. Dewan Y, Komolafe EO, Mejía-Mantilla JH, et al. CRASH-3-tranexamic acid for the treatment of significant traumatic brain injury: study protocol for an international randomized, double-blind, placebo-controlled trial. *Trials* 2012; 13: 87.
8. Kim JJ, Gean AD. Imaging for the diagnosis and management of traumatic brain injury. *Neurotherapeutics* 2011;8: 39-53.
9. Townend W, Ingebrigtsen T. Head injury outcome prediction: a role for protein S-100B? *Injury* 2006; 37: 1098-1108.
10. Rainey T, Lesko M, Sacho R, et al. Predicting outcome after severe traumatic brain injury using the serum S100B biomarker: results using a single (24 h) time-point. *Resuscitation* 2009; 80: 341-345.
11. Mabe H, Suzuki S, Mase M, et al. Serum neuron-specific enolase levels after subarachnoid haemorrhage. *Surg Neurol* 1991; 36: 170-174.
12. Meric E, Gunduz A, Turedi S, et al. The prognostic value of neuron-specific enolase in head trauma patients. *J Emerg Med* 2010 ;38: 297-301.
13. Bandyopadhyay S, Hennes H, Gorelick MH, et al. Serum neuron - specific enolase as a predictor of short - term outcome in children with closed traumatic brain injury. *Acad Emerg Med* 2005; 12: 732-738.
14. Cheng F, Yuan Q, Yang J, et al. The prognostic value of serum neuron-specific enolase in traumatic brain injury: systematic review and meta-analysis. *PloS One* 2014; 9: e106680.

ORIGINAL ARTICLE

Effect of hypothyroidism on haematological parameters: a gender- based comparison

Samia Karkoutly; Taghrid Hammoud and Faizeh Al-Quobaili

ABSTRACT

Background: Hypothyroidism is one of the most frequently diagnosed thyroid disorders, especially in women. Prevalence of anaemia in subclinical and overt hypothyroid patients is 26.6 % and 73.2 %, respectively. Thus, haematological abnormalities are frequent in hypothyroid patients, but rarely investigated.

Objectives: The purpose of the current study was to investigate the effect of hypothyroidism on haematological parameters, to assess potential associations between these parameters and thyroid hormones, and to evaluate gender-based differences of haematological parameters in hypothyroid patients.

Methods: This cross-sectional retrospective study included 54 newly diagnosed, untreated hypothyroid patients and 30 healthy individuals. Patients with anaemia, endocrine disorders other than hypothyroidism and chronic liver, kidney, heart or inflammatory diseases were excluded. Thyroid stimulation hormone (TSH) and free thyroxine (FT4) levels were determined and haematological parameters were compared between the two groups.

Results: Haemoglobin, haematocrit, mean corpuscular volume (MCV), and mean corpuscular haemoglobin (MCH) levels were significantly lower, and red blood cell distribution width (RDW) levels were significantly higher in the hypothyroid group compared to the control group. Furthermore, red blood cell count (RBC), haemoglobin, haematocrit, MCV and MCH levels were significantly lower and RDW levels were significantly higher in female patients compared to male patients. A weak but significant correlation was observed between TSH and RDW in the hypothyroid group (r=+0.44; *p*=0.001).

Conclusions: Hypothyroidism has a direct effect on most haematology parameters. These effects were more prominent in female patients.

Keywords: Primary hypothyroidism; thyrotropin; erythrocyte indices; gender.

N Z J Med Lab Sci 2020; 74: 98-102.

INTRODUCTION

Thyroid disorders, are the most frequently diagnosed endocrine disorders (1), are a global health problem with serious health consequences (2). Hypothyroidism is the most encountered thyroid disorder (3) with a prevalence between 0.6 and 12 per 1000 in women and between 1.3 and 4.0 per 1000 in men (4). Thus, it is approximately 5–10 times more often diagnosed among females than males (2). The great majority of patients have the primary form of the disease (3). Hypothyroidism is a condition resulting from an absence or deficiency of thyroid hormones which may have devastating consequences (5). For example, untreated hypothyroidism can contribute to hypertension, infertility and many other complications (6), including anaemia (7).

The prevalence of subclinical and primary hypothyroidism is constantly increasing, especially in women (8). Previous animal experiments (9-11) and studies on human cell lines (12) and human subjects (13-15) have indicated that anaemia is a common finding in patients with hypothyroidism. Prevalence of anaemia in subclinical and overt hypothyroid patients is 26.6% and 73.2%, respectively (14). In 2008, a study on mice deficient for thyroid hormone receptor α (TRα) showed that adult deficient mice had lower haematocrit levels and an altered stress erythropoiesis response to haemolytic anaemia (9). In 2009 a randomised, double-blind controlled study indicated that iron-deficiency anaemia did not respond to oral iron therapy adequately in subclinical hypothyroid patients. However, the addition of levothyroxine caused a significant improvement in serum iron and blood count (16). In 2010 a direct cause-and-effect association between thyroid disorders and human haematopoiesis was revealed (17).

Although there are several studies regarding the association between anaemia and hypothyroidism, information is scant about possible correlations between thyroid hormones and haematological parameters. To our knowledge there are no published studies that describe gender-based differences in haematological parameters in hypothyroid patients.

The aim of this study was to investigate the effect of hypothyroidism on haematological parameters, to assess potential associations between these parameters and thyroid hormones and to evaluate gender-based differences of haematological parameters in hypothyroid patients.

MATERIALS AND METHODS

A total of 84 subjects were recruited in this cross-sectional retrospective study. We categorize these subjects into two groups according to thyroid status. The first group (hypothyroid group) consisted of 54 newly diagnosed, untreated hypothyroid patients aged between 20 and 60 years. These patients presented to endocrinology outpatient clinic at Al Assad University Hospital, Damascus, Syria. The second group (control group) consisted of 30 healthy, age and gender-matched subjects. Hypothyroidism was defined as thyroid stimulating hormone (TSH) levels >10mIU/L and free thyroxine (FT4) levels <7pmol/L, based on Clinical Practice Guidelines for Hypothyroidism in Adults by the American Association of Clinical Endocrinologists and the American Thyroid Association (18). Studied haematological parameters were red blood cell count (RBC), haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red blood cell distribution width (RDW).

Individuals were excluded from the study if they presented with chronic liver, kidney or heart diseases, inflammatory diseases, asthma, malignancy, prior thyroid disorders and/or treatment history, diabetes mellitus or any endocrine system disease other than hypothyroidism. Additional exclusion criteria included haemoglobinopathies, nutritional deficiency anaemia and pregnancy.

After collecting baseline characteristics and medical history of the patients by interviewing in the clinic, samples were collected in the fasting state.

About 6 ml of venous blood was collected, in which 3 ml blood was taken on EDTA and remaining 3 ml centrifuged to separate serum. Haemolyzed and lipemic samples were discarded. EDTA samples were subjected for complete blood count using the Abbott Cell Dyn 3500 haematology analyzer (Abbott, Inc., Germany). Serum TSH and FT4 levels were assessed by enzyme-linked immunosorbent assay (TSH ELISA kit and FT4 ELISA kit, DIAsource, Inc., Belgium).

The research project was approved by the Ethics Committee and registered at Damascus University. Informed consent was obtained from each participant prior to enrolment in the study. All the subjects' information was kept confidential.

Statistical analysis was performed by SPSS 22.0 (SPSS, Inc., Chicago, USA) packed software for windows. Data are expressed as the mean ± standard deviation. Differences between groups were tested by t-student test for normally distributed data and Mann-Whitney test for non-normally distributed data. The χ^2 test was performed for categorical data comparisons. The linear association between the quantitative variables was examined using the Pearson's correlation test or Spearman's correlation test, depending on data distribution. The level of statistical significance was set at $p<0.05$.

RESULTS

In total 84 subjects were assessed. 54 patients were included in the hypothyroid group and 30 healthy subjects in the control group. Age showed no statistically significant difference between groups (p = 0.272). 44 of 54 patients in the hypothyroid group (81.5 %) and 21 of 30 subjects in the control group (70.0 %) were female. Gender was not significantly different between the groups (P = 0.228).

No statistically significant differences were observed in terms of RBC and MCHC ($p>0.05$) between the hypothyroid and control groups. However, haemoglobin, haematocrit, MCV, and MCH levels were significantly lower in the hypothyroid group compared to the control group ($p<0.05$). In addition, RDW levels in the hypothyroid group were significantly higher compared to the control group ($p<0.001$).

Age, TSH levels, FT4 levels and MCHC showed no statistically significant differences ($p>0.05$) between the groups (Table 2). Conversely the RBC count, haemoglobin, haematocrit, MCV and MCH levels were significantly lower in female patients compared to male patients ($p<0.05$). RDW levels were significantly higher in female patients compared to male patients ($p<0.05$).

Correlations between TSH and haematological parameters were investigated (Table 3 and Figure 1). A weak but significantly positive correlation was observed between TSH and RDW in hypothyroid group (r= +0.44; p=0.001) (Figure 1). However, other haematological parameters did not exhibit statistically significant correlation with TSH. There was no statistically significant correlation identified between FT4 and haematological parameters in both the hypothyroid and control groups (Table 4).

Table 1. General characteristics and laboratory data of control subjects and hypothyroid patients

Parameters	Control (n=30)	Hypothyroid (n=54)	*p**
Gender (male/female)	9/21	10/44	0.181
Age (years)†	47 ± 13.5	42.1 ± 13.1	0.421
RBC ($x10^{12}$/L)†	4.7 ± 0.2	4.6 ± 0.2	0.176
Haemoglobin (g/L)‡	133 ± 6	122 ± 12	0.0001
Haematocrit (L/L)†	0.42 ± 0.01	0.38 ± 0.03	0.0001
MCV (fl)‡	88.1 ± 2.7	82.0 ± 6.2	0.0001
MCH (pg)‡	28.2 ± 1.1	25.4 ± 2.8	0.0001
MCHC (g/L)‡	320 ± 6.0	318 ± 17	0.159
RDW (%)‡	13.5 ± 0.6	16.0 ± 2.2	0.0001
TSH (mIU/L)†	1.7 ± 0.8	16.7 ± 6.3	0.0001
FT4 (pmol/L)‡	11.4 ± 2.2	5.1 ± 0.8	0.0001

Data are presented as mean ± standard deviation; *The level of statistical significance was set at $p<0.05$; † refers to Mann-Whitney test; ‡ refers to T-student test; TSH, thyroid stimulating hormone; FT4, free thyroxine; RBC, red blood cell; MCV, mean-orpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red blood cell distribution width.

Table 2. General characteristics and laboratory data of hypothyroid patients differentiated by gender

Parameters	Male (n=10)	Female (n=44)	*p**
Age (years)†	47 ± 10.9	40.6 ± 13.2	0.228
RBC ($x10^{12}$/L)†	4.8 ± 0.2	4.4 ± 0.2	0.032
Haemoglobin (g/L)‡	124 ± 20	117 ± 12	0.016
Haematocrit (L/L)†	0.42 ± 0.06	0.38 ± 0.03	0.022
MCV (fl)‡	85.2 ± 4.8	82.2 ± 7.0	0.028
MCH (pg)‡	27.3 ± 2.1	25.8 ± 3.1	0.023
MCHC (g/L)‡	319 ± 9.0	314 ± 16	0.089
RDW (%)‡	15.9 ± 2.0	17.0 ± 2.2	0.036
TSH (mIU/L)†	18.6 ± 6.4	16.2 ± 6.1	0.134
FT4 (pmol/L)‡	5.3 ± 0.8	5.1 ± 2.2	0.207

Data are presented as the mean ± standard deviation; *The level of statistical significance was set at $p<0.05$; † refers to T-student test; ‡ refers to Mann-Whitney test; TSH, thyroid stimulating hormone; FT4, free thyroxine; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red blood cell distribution width.

Table 3. Correlation coefficients of TSH with haematological parameters in the control and hypothyroid groups

	Control group (n=30)		Hypothyroid group (n=54)	
	r	*p*	r	*p*
RBC (x10^{12}/L)	-0.179	0.345	-0.048	0.730
Haemoglobin (g/L)	-0.069	0.717	-0.253	0.065
Haematocrit (L/L)	-0.098	0.605	-0.247	0.072
MCV (fl)	0.057	0.766	-0.152	0.271
MCH (pg)	-0.033	0.863	-0.279	0.065
MCHC (g/L)	-0.235	0.211	-0.162	0.389
RDW (%)	0.371	0.058	0.438	0.001

TSH,thyroid stimulating hormone; r, correlation coefficient; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red blood cell distribution width.

Table 4. Correlation coefficients of FT4 With haematological parameters in the control and hypothyroid groups

	Control group (n=30)		Hypothyroid group (n=54)	
	r	*p*	r	*p*
RBC (x10^{12}/L)	0.105	0.580	0.008	0.954
Haemoglobin (g/L)	0.162	0.391	0.036	0.794
Haematocrit (L/L)	0.229	0.223	0.059	0.671
MCV (fl)	0.192	0.310	0.031	0.823
MCH (pg)	0.186	0.326	0.003	0.985
MCHC (g/L)	0.077	0.684	0.069	0.618
RDW (%)	-0.086	0.651	-0.221	0.067

FT4, free thyroxine; r, correlation coefficient; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red blood cell distribution width.

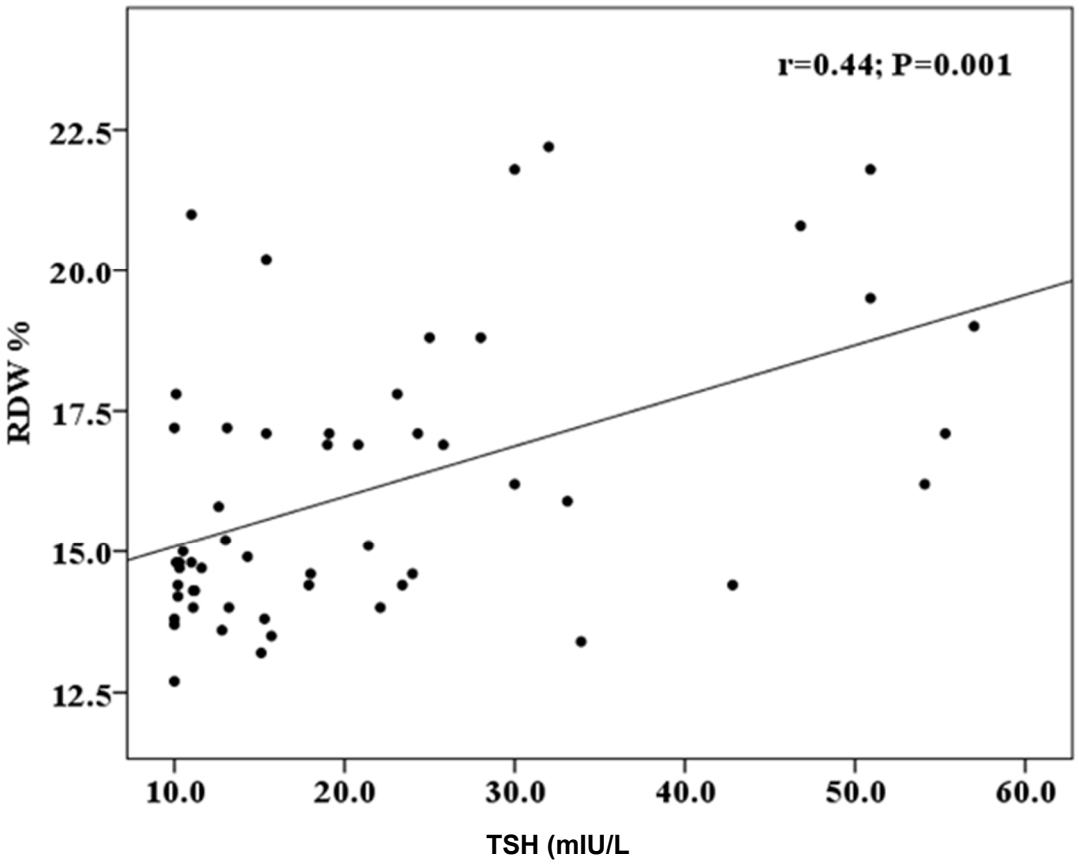


Figure 1. Correlation between TSH levels and RDW in hypothyroid group (n=54). TSH was positively correlated with RDW (r= +0.44; *P*=0.001). TSH, thyroid stimulating hormone; RDW, red blood cell distribution width; r, Spearman's rho.

DISCUSSION

To our knowledge, this is the first study on assessing gender-based differences of haematological parameters in patients with hypothyroidism. According to obtained data, most haematological parameters were significantly lower in female than in male patients, except for RDW levels which were significantly higher in females. These results are in line with Kulkarni *et al.,* which concluded that the prevalence of anaemia in hypothyroid patients is higher among females (14). Our data suggests that in hypothyroidism, females are more prone to erythrocyte abnormalities than males. These abnormalities may develop due to menorrhagia occurring as a result of various hormonal instability and malabsorption observed in hypothyroidism (7,19).

Our results show that haematocrit and haemoglobin levels were significantly lower in the hypothyroid group, compared to the control group, which is in agreement with Mehmet *et al.*, Dorgalaleh *et al.* and Kulkarni *et al.* (7,14,20). However, unlike our findings, Olt *et al.,* reported that no significant difference was observed between hypothyroid and control groups regarding haemoglobin levels (21). The differences in results presented could be explained by the small number of subjects included in the Olt *et al.* study (20 hypothyroid patients and 22 healthy subjects).

According to our data, MCV levels were significantly lower in hypothyroid group compared to control group, suggesting that our patients were at the risk of microcytic anaemia. This finding is similarly reported by Dorgalaleh *et al.* (20). On the contrary, Bashir *et al.* and Geetha *et al.* found that MCV levels were significantly higher in patients with hypothyroidism (8,22). The present study is also not in accordance with Mehmet *et al.* and Aktas *et al.* which concluded that there was no significant difference in MCV levels between healthy subjects and hypothyroid patients (7,23). This variation in results is consistent with the literature. Different forms of anaemia might be encountered in thyroid dysfunction patients, depending on comorbidities (24). Normochromic normocytic, hypochromic microcytic and macrocytic types are all reported by different authors (13,19).

Data of our study demonstrated that RDW levels were significantly higher in hypothyroid patients, compared to healthy subjects. Anisocytosis in hypothyroid patients was also found by Serdar Olt *et al.*, Bashir *et al.*, Dorgalaleh *et al.* and Geetha *et al.* (8,19,21,22). A study by Aktas *et al.* demonstrated that elevated RDW could be a marker in Hashimoto's thyroiditis (23). Additionally, a weak, but significantly positive correlation between RDW and TSH levels was observed in our study in the hypothyroid group. This correlation is consistent with Yu *et al.* which is the only study thus far that has assessed the correlation between RDW and TSH in subclinical hypothyroid patients (25). In the Yu *et al.* study, subjects were classified into two groups based on TSH levels representing a euthyroid state and subclinical hypothyroidism (25). These two groups were further subdivided into four TSH groups and the association between RDW and TSH was studied. In all four groups, TSH levels were significantly correlated with RDW. Interestingly, when TSH>7.5 uIU/ml, the strongest association between RDW and TSH levels was observed (r=+0.166; p=0.006). Erythropoietin is one of the major determinants of RDW. It was shown that abnormal erythropoietin production may induce a gradual increase in RDW levels (26). Furthermore, hypothyroid patients show a decreased plasma levels of erythropoietin (13). In a randomised controlled trial by Christ-Crain *et al.,* erythropoietin levels increased significantly after levothyroxine replacement therapy in female patients with subclinical hypothyroidism (27). Although the exact mechanisms explaining the association between RDW and thyroid diseases have not been defined yet, we may conclude that elevated RDW values in hypothyroidism might be resulting from abnormal erythropoietin levels.

Thyroid hormones may have a direct effect on bone marrow by influencing erythroid precursor proliferative capacity and no other lineages (28-30). In addition, thyroid hormones may affect erythropoiesis indirectly by inducing gene expression and kidney secretion of erythropoietin (20,28). However, the exact mechanisms by which thyroid hormones modulate erythropoiesis is currently unclear (28).

Our study had several limitations. First, the retrospective design of the study. Second, the small number of patients included in our study was an obstacle, especially in having a more reliable statistical analysis. Third, no measurements for ferritin or serum iron were available. For these reasons, prospective larger studies would be useful to provide additional information regarding the relationship between hypothyroidism and haematological parameters.

CONCLUSION

In conclusion, our study showed that hypothyroidism has a direct effect on most haematological parameters and these effects were more prominent in female patients. In addition, a significant correlation between TSH and RDW was demonstrated in the hypothyroid group. Moreover, our study suggests that hypothyroid patients are at a greater risk of having lower haemoglobin values, compared to the general population. We suggest that hypothyroid patients, especially women, should periodically have haematological parameters investigated for possible changes.

ACKNOWLEDGEMENTS

We would like to thank all the laboratory staff, nurses, residents and consultants from the Department of Endocrinology at Al-Assad University Hospital. We would also like to thank the administration of Damascus University for financial support.

AUTHOR INFORMATION

Samia Karkoutly, MSc, Staff Researcher[1]
Taghrid Hammoud, MD PhD, Associate Professor[2,3]
Faizeh Al Quobaili, PhD, Professor and Dean[4]

[1]Department of Biochemistry and Microbiology, Faculty of Pharmacy, Damascus University, Damascus, Syria
[2]Department of Physiology and Pharmacology, Faculty of Medicine, Damascus University, Damascus, Syria
[3]Department of Internal Medicine, Endocrinology Service, Al-Mouassa Hospital, Damascus, Syria
[4]Department of Clinical Biochemistry and Microbiology, Faculty of Pharmacy, Damascus University, Syria

Correspondence: Samia Karkoutly.
Email: samiakarkoutly@hotmail.com

REFERENCES

1. Matyjaszek-Matuszek B, Pyzik A, Nowakowski A, Jarosz MJ. Diagnostic methods of TSH in thyroid screening tests. *Ann Agric Environ Med* 2013; 20: 731-735.
2. Taylor PN, Albrecht D, Scholz A, Gutierrez-Buey G, Lazarus JH, Dayan CM, et al. Global epidemiology of hyperthyroidism and hypothyroidism. *Nat Rev Endocrinol* 2018; 14: 301-316.
3. Curl K-AG. Hypothyroidism: clinical challenges in diagnosis and treatment. *Clinician Reviews* 2014; 24: 40-46.
4. Almandoz JP, Gharib H. Hypothyroidism: etiology, diagnosis, and management. *Med Clin North Am* 2012; 96: 203-221.
5. Hershman JM. Hypothyroidism and hyperthyroidism. In: N Lavin, Editor. Manual of Endocrinology and Metabolism. 5th ed. Wolters Kluwer, Philadelphia, USA, 2019: 510-522.
6. Gaitonde DY, Rowley KD, Sweeney LB. Hypothyroidism: an update. *Am Fam Physician* 2012; 86: 244-251.
7. Erdogan M, Kösenli A, Ganidagli S, Kulaksizoglu M. Characteristics of anemia in subclinical and overt hypothyroid patients. *Endocr J* 2012; 59: 213-220.

8. Bashir H, Bhat MH, Farooq R, Majid S, Shoib S, Hamid R, et al. Comparison of hematological parameters in untreated and treated subclinical hypothyroidism and primary hypothyroidism patients. *Med J Islam Repub Iran* 2012; 26: 172-8.
9. Kendrick TS, Payne CJ, Epis MR, Schneider JR, Leedman PJ, Klinken SP, et al. Erythroid defects in TRalpha-/- mice. *Blood*. 2008; 111: 3245-3248.
10. Zhang Y, Xue Y, Cao C, Huang J, Hong Q, Hai T, et al. Thyroid hormone regulates hematopoiesis via the TR-KLF9 axis. *Blood* 2017; 130: 2161-2170.
11. Park S, Han CR, Park JW, Zhao L, Zhu X, Willingham M, et al. Defective erythropoiesis caused by mutations of the thyroid hormone receptor α gene. *PLoS Genet* 2017; 13: .
12. Ma Y, Freitag P, Zhou J, Brüne B, Frede S, Fandrey J. Thyroid hormone induces erythropoietin gene expression through augmented accumulation of hypoxia-inducible factor-1. *Am J Physiol Regul Integr Comp Physiol*. 2004; 287: R600-R607.
13. Szczepanek-Parulska E, Hernik A, Ruchała M. Anemia in thyroid diseases. *Pol Arch Intern Med* 2017; 127: 352-360.
14. Kulkarni VK, Jadhav DU. A study of anemia in primary hypothyroidism. *Int J Adv Med* 2017; 4: 383-389.
15. Das C, Sahana PK, Sengupta N, Giri D, Roy M, Mukhopadhyay P. Etiology of anemia in primary hypothyroid subjects in a tertiary care center in Eastern India. *Indian J Endocr Metab* 2012; 16(Suppl 2): S361-3.
16. Cinemre H, Bilir C, Gokosmanoglu F, Bahcebasi T. Hematologic effects of levothyroxine in iron-deficient subclinical hypothyroid patients: a randomized, double-blind, controlled study. *J Clin Endocrinol Metab* 2009; 94: 151-156.
17. Kawa MP, Grymula K, Paczkowska E, Baskiewicz-Masiuk M, Dabkowska E, Koziolek M, et al. Clinical relevance of thyroid dysfunction in human haematopoiesis: biochemical and molecular studies. *Eur J Endocrinol* 2010; 162: 295-305.
18. Garber JR, Cobin RH, Gharib H, Hennessey JV, Klein I, Mechanick JI et al. Clinical practice guidelines for hypothyroidism in adults: cosponsored by The American Association of Clinical Endocrinologists and The American Thyroid Association. *Endocr Pract*. 2012; 18: 988-1028. Erratum in: *Endocr Pract* 2013 ;19: 175.
19. Soliman AT, De Sanctis V, Yassin M, Wagdy M, Soliman N. Chronic anemia and thyroid function. *Acta Biomed* 2017; 88: 119-127.
20. Dorgalaleh A, Mahmoodi M, Varmaghani B, Kiani Node F, Saeeidi Kia O, Alizadeh Sh, et al. Effect of thyroid dysfunctions on blood cell count and red blood cell indice. *Iran J Ped Hematol Oncol* 2013; 3: 73-77.
21. Olt S, Selçuk M, Tutak A, Akbaş F, Öznas O. Investigation the impact of hypothyroidism on hematological parameters. *Mustafa Kemal Üniv Tıp Derg* 2016; 7: 23-27.
22. Geetha JP, Srikrishna R. Role of red blood cell distribution width (RDW) in thyroid dysfunction. *Int J Biol Med Res* 2012; 3: 1476-1478.
23. Aktas G, Sit M, Dikbas O, Tekce BK, Savli H, Tekce H, et al. Could red cell distribution width be a marker in Hashimoto's thyroiditis? *Exp Clin Endocrinol Diabetes* 2014; 122: 572–574.
24. Floriani C, Feller M, Aubert CE, M'Rabet-Bensalah K, Collet TH, den Elzen WPJ, et al. Thyroid dysfunction and anemia: a prospective cohort study and a systematic review. *Thyroid* 2018; 28: 575-582.
25. Yu HM, Park KS, Lee JM. The value of red blood cell distribution width in subclinical hypothyroidism. *Arq Bras Endocrinol Metabol* 2014; 58: 30-36.
26. Salvagno GL, Sanchis-Gomar F, Picanza A, Lippi G. Red blood cell distribution width: a simple parameter with multiple clinical applications. *Crit Rev Clin Lab Sci* 2015; 52: 86-105.
27. Christ-Crain M, Meier C, Huber P, Zulewski H, Staub JJ, Müller B. Effect of restoration of euthyroidism on peripheral blood cells and erythropoietin in women with subclinical hypothyroidism. *Hormones (Athens)* 2003; 2: 237-242.
28. Wopereis DM, Du Puy RS, van Heemst D, Walsh JP, Bremner A, Bakker SJL, et al. The relation between thyroid function and anemia: a pooled analysis of individual participant data. *J Clin Endocrinol Metab* 2018; 103: 3658-3667.
29. Schindheim RK, ten Boekel E, Heima NE, Schoor NM, Simsek S. Thyroid hormones and erythrocyte indices in a cohort of euthyroid older subjects. *Eur J Intern Med* 2013; 24: 241–244.
30. Bremner A, Feddema P, Joske D, Leedman P, O'Leary P, Olynyk J, et al. Significant Association Between Thyroid Hormones and Erythrocyte Indices in Euthyroid Subjects. *Clin Endocrinol* 2012; 76: 304–311.

Journal reviewers August 2019 to July 2020

The persons listed below reviewed submitted articles for the Journal between August 2019 and July 2020, some more than once. All submitted articles undergo peer review in order that the Journal maintains its high standard. Additionally, thoughtful comments and suggestions made by reviewers help authors in ensuring that their articles, if accepted, are put in front of the reader in the best possible light. The Editors thanks the reviewers for their valuable time and effort.

REVIEWERS

Paul Austin, Auckland
Dr. Collette Bromhead, Wellington
A/Prof. Heather Brooks, Dunedin
Julie Creighton, Christchurch
Dr. Joanne Hewitt, Porirua
Chris Kendrick, Palmerston North
A/Prof. Michael Legge, Dunedin
Craig Mabbett, New Plymouth
Dennis Mok, Australia
Michelle Petrasich, Auckland
Beverley Pullon, Hamilton
Prof. Mohd Shahid, Bahrain
A/Prof. Rob Siebers, Wellington
Terry Taylor, Dunedin

ORIGINAL ARTICLE

The process control, design and planning stage of ISO 15189:2012 management system standard: an implementation update

Dennis Mok and Sharfuddin Chowdhury

ABSTRACT

Objectives: The primary aim of this paper was to provide an update on selected internationally-oriented guidance documents and relevant literature at the application level that are associated with the implementation of the 'process control, design and planning' stage of ISO 15189:2012.

Methods: Additional relevant internationally-oriented guidance documents were identified from the Union of International Associations segmented international organisations (Type A to Type F) and supplemented with additional literature.

Results: Selected international organisations (*n* = 28), inclusive of additional organisations (*n* = 16), were found to provide relevant internationally-oriented guidance documents (*n* = 237) in support of implementation of the process control, design and planning stage of ISO 15189:2012. An updated list of literature (*n* = 223) was also provided for further reference.

Conclusions: The present study contributes to the medical laboratory's development of implementations of ISO 15189:2012 in areas of operations by fulfilling management system and technical competence requirements to an acceptable level of conformance that are regulated by managerial and technical specifications.

Key words: clinical competence, conformity, ISO 15189:2012, quality improvement, total quality management.

N Z J Med Lab Sci 2020; 74: 103-106.

INTRODUCTION

The contemporary medical laboratories optimise their services by improving processes in the areas of operations by implementing relevant management system standards, such as ISO 15189:2012 management system standard (1) developed by the International Organization for Standardization (ISO) (2,3,pp.152-153). The implementation of ISO 15189:2012 remains the management system standard of choice for the human pathology services industry and offers the possibility of accreditation by an authorised organisation for the medical laboratory (4).

According to an ISO 15189:2012 process-based quality management system model (5), the 'process control, design and planning (PCDP)' stage of ISO 15189:2012 comprises one subclause from Clause 4 (Management requirements) of ISO 15189:2012 (1,pp.6-19) and four subclauses from Clause 5 (Technical requirements) of ISO 15189:2012 (1,pp.19-39). It has also been determined that the PCDP stage of ISO 15189:2012 contains 21/119 (18 %) administrative requirements (6) and 477/1515 (31 %) conformance requirements (CRs) (5). The PCDP stage of ISO 15189:2012 fulfils a vital role in providing managerial and technical specifications that support the analytical processes for routine medical laboratory operations. The specifications can be clarified and simplified by using relevant guidance documents covering the medical laboratory operational processes.

The main challenge of the implementation of PCDP stage of ISO 15189:2012 is to ensure all specifications are compatible among the analytical process infrastructure. The level of compatibility acceptance can be relatively complicated that is largely depending upon the range of service provision. A balanced and well regulated mix of specifications can attain the required concentration of analytical effort and support effectiveness as well as efficiency.

This paper provides an update on internationally-oriented guidance documents associated with the PCDP stage of ISO 15189:2012 at the application level in the areas of interest, especially in marking information, that may require reasonably practicable effort for the fulfilment of CRs. This update should be used in conjunction with the previously published 'ISO 15189:2012 implementation: an update of related international guidance documents for medical laboratory quality management' in the *N Z J Med Lab Sci* (7) and 'The strategic management stage of ISO 15189:2012 management system standard: an implementation update' in the *N Z J Med Lab Sci* (8). Overall, this update provides relevant information for medical laboratories on the implementation of subclauses relating to the PCDP stage of ISO 15189:2012.

MATERIALS AND METHODS

Selection criteria of international organisations International organisations that were accepted by the Union of International Associations as either Type A, B, C, D, E or F (9,pp.xv-xxi) and published in 'Yearbook of international organizations 2019 - 2020: guide to global civil society networks' (9) were selected for inclusion. Internationally-oriented national organisations, classified as Type G by the Union of International Associations, were excluded for the selection.

Selection of recommended guidance documents associated with the process control, design and planning stage of ISO 15189:2012

This update focused on the PCDP stage of ISO 15189:2012 published by the ISO. The subclauses of interest were Subclauses 4.6 (External services and supplies) (1,pp.12-13), 5.1 (Personnel) (1,pp.19-21), 5.2 (Accommodation and environmental conditions) (1,pp.21-23), 5.3 (Laboratory equipment, reagents, and consumables) (1,pp.23-26) and 5.10 (Laboratory information management) (1,pp.38-39) of ISO 15189:2012. Selected internationally-oriented guidance documents that could provide reasonable support to the abovestated subclauses of interest were selected for inclusion.

Selection of recommended guidance documents associated with suggested environmental factors in Subclause 5.2.6 (Facility maintenance and environmental conditions) of ISO 15189:2012

Selected internationally-oriented guidance documents that could provide practical support to manage environmental factors relating to the health of personnel and the quality of results in the examination process were selected for inclusion.

Selection of relevant literature associated with the process control, design and planning stage of ISO 15189:2012

Additional resources associated with the PCDP stage of ISO 15189:2012 were selected for inclusion. Literature listed in the previous update (7) is excluded in this update.

RESULTS

Selected international organisations providing relevant guidance documents

This update includes additional international organisations (*n* = 16) that provide relevant guidance documents for the implementation of the PCDP stage of ISO 15189:2012 (Table 1).

Recommended guidance documents associated with the process control, design and planning stage of ISO 15189:2012

This update has identified internationally-oriented guidance documents (*n* = 293) to provide relevant information for the implementation of the PCDP stage of ISO 15189:2012; containing the listed documents (*n* = 237) (Table S2) and the training content of health and safety requirements related documents in Subclause 5.1.4 (Personnel introduction to the organizational environment) of ISO 15189:2012 (1,p.20) (*n* = 57) (see ‡‡).

Recommended guidance documents associated with the influence of health of personnel in the medical laboratory in Subclause 5.2.6 (Facility maintenance and environmental conditions) of ISO 15189:2012

Relevant internationally-oriented guidance documents that were able to provide support to environmental factors (*n* = 18) that may adversely influence the health of personnel were identified (*n* = 75) and linked to related factors (Table S3).

Table 1. Additional international organisations providing relevant guidance documents in support of the implementation of process control, design and planning stage of ISO 15189:2012.

Organisations (*n* = 16)	**Classification** (Type A to Type F)
European Committee for Standardization (CEN)	D
European Committee for Electrotechnical Standardization (CENELEC)	D
International Commission on Illumination (CIE)	C
Cooperative on International Traceability in Analytical Chemistry (CITAC)	F
Eurachem	F
International Agency for Research on Cancer (IARC)	E
International Air Transport Association (IATA)	B
International Commission on Non-Ionizing Radiation Protection (ICNIRP)	D
International Commission on Occupational Health (ICOH)	B
International Social Security Association (ISSA)	B
International Solid Waste Association (ISWA)	B
International Union of Pure and Applied Chemistry (IUPAC)	B
Organisation for Economic Co-operation and Development (OECD)	C
Transparency International (TI)	F
United Nations Economic Commission for Europe (UNECE)	E
WHO Regional Office for Europe (WHO/Europe)	E

Descriptions (9, pp. xv-xxi)**:**

Type B: universal membership organisation: from either ≥ 60 countries or ≥ 30 countries in ≥ two continents and with a well-balanced geographical distribution; management and policy-making organs reflect a well-balanced geographical distribution.

Type C: intercontinental membership organisation: from ≥ 10 countries in ≥ two continents with a well-balanced geographical distribution; management and policy-making organs reflect a well-balanced geographical distribution.

Type D: regionally defined membership organisation: from ≥ three countries within one continental or sub-continental region; management and policy-making organs reflect a well-balanced geographical distribution.

Type E: organisation emanating from places, persons or other bodies: no criteria for membership; reference to, and to some degree limited by, another international organisation, or a person, or a place.

Type F: organisation having a special form: no criteria for membership; structure is non-formal, unconventional or unusual.

Recommended guidance documents associated with the influence of the quality of results in the medical laboratory in Subclause 5.2.6 (Facility maintenance and environmental conditions) of ISO 15189:2012

Relevant internationally-oriented guidance documents that were able to provide support to environmental factors (*n* = 11) that may adversely influence the quality of results in the examination process were identified (*n* = 47) and linked to related factors (Table S4).

Relevant literature associated with the process control, design and planning stage of ISO 15189:2012

Additional references (*n* = 223) that were found to provide further relevant information for the implementation of the PCDP stage of ISO 15189:2012 were identified (Table S5).

DISCUSSION

Process control, design and planning stage of ISO 15189:2012

The PCDP stage of ISO 15189:2012 comprises Subclauses 4.6, 5.1, 5.2, 5.3 and 5.10 of ISO 15189:2012, containing 477/1515 (31 %) CRs. Additional international organisations (Table 1) were identified to provide further guidance documents to support the implementation. The completed list of selected international organisations (Table S1), recommended guidance document (Table S2) and additional resources (Table S5) that could support the implementation are presented. This update is recommended to use in conjunction with the previously published updates (7, 8).

This update has additional guidance notes to support the internal auditors to interpret information to ensure fulfilments are consistent with classification and type according to the organisation's specifications. First, marking information was selected and summarised in footnotes (see † to §§§§§§). The term 'marking' has been defined by the International Electrotechnical Commission and the ISO as 'symbols, pictograms, warnings, logos, or inscriptions on the consumer product, label or packaging to identify its type, which can also include short textual messages' in Subclause 3.12 of ISO/IEC Guide 14:2018 (10,p.2). Second, safety information was selected and relevant subclauses indicated in footnotes (see † to §§§§§§).

Subclause 4.6 (External services and supplies) of ISO 15189:2012

Subclauses 4.6 of ISO 15189:2012 specifies that the medical laboratory must manage externally provided processes, products and services by the implementation of a documented procedure.

Implications for implementers: no additional notes. *Implications for internal auditors*: no additional notes.

Subclause 5.1 (Personnel) of ISO 15189:2012

Subclauses 5.1 of ISO 15189:2012 specifies that the medical laboratory must implement personnel management practices by the implementation of a documented procedure. *Implications for implementers*: the medical laboratory should management personnel with the support of personnel who are qualified in human resource management when it is operationally feasible and effective to do so. Human resource management is a specialised area and it is highly unlikely that laboratory management is aware of all human resource management implications during the implementation. It is also important that the training content of health and safety requirements in Subclause 5.1.5 d) of ISO 15189:2012 (1,p.20) should be in alignment with the content of Subclause 5.1.4 of ISO 15189:2012. This is to ensure consistent flow of information is delivered to managerial and technical personnel.

Implications for internal auditors: the indernal audit process must ensure the internal audits are conducted by personnel who are properly trained and competent in internal auditing. The internal auditors must have the applicable practical and theoretical background to practise, as specified in Subclause 5.1.2 (Personnel qualifications) of ISO 15189:2012 (1,p.19).

Subclause 5 .2 (Accommodation and environmental conditions) of ISO 15189:2012

Subclause 5.2 of ISO 15189:2012 specifies that the medical laboratory must be well maintained with reasonable effort with specified environmental conditions to provide effective operations.

Implications for implementers: the medical laboratory must monitor, control and record relevant environmental conditions, as specified in Subclause 5.2.6 (Facility maintenance and environmental conditions) of ISO 15189:2012 (1,p.23). However, suggested factors listed in Subclause 5.2.6 of ISO 15189:2012 should be considered as a priority. Suggested factors that may influence the health of personnel (Table S3) and the quality of results in the examination process (Table S4) are linked to relevant guidance documents for consideration.

Implications for internal auditors: the internal audit process must ensure appropriate acceptable ranges are used from relevant guidance documents. It is also important to note that these ranges must be in alignment with national and regional requirements pertaining to the particular activity.

Subclause 5.3 (Laboratory equipment, reagents, and consumables) of ISO 15189:2012

Subclause 5.3 of ISO 15189:2012 specifies that the medical laboratory must manage laboratory equipment, reagents and consumables by the implementation of documented procedures.

Implications for implementers: the medical laboratory must have adequate storage capability for flammable liquids that require refrigerated condition (2 °C to 8 °C), such as the use of 'explosion-safe' non-sparking refrigerators, to fulfil storage and handling to fulfil CRs of Subclause 5.3.2.2 (Reagents and consumables — Reception and storage) of ISO 15189:2012 (1,p.25).

Implications for internal auditors: no additional notes.

Subclause 5.10 (Laboratory information management) of ISO 15189:2012

Subclause 5.10 of ISO 15189:2012 specifies that the medical laboratory must implement an effective laboratory information management system to support the medical laboratory service operations.

Implications for implementers: the medical laboratory should deploy robust security techniques for the information security management system, especially in storage security. Appropriate level of attention should be paid to address the emerging susceptibility and vulnerability issues of the laboratory information management system by implementing effective firewalls (11) to guard against cybercriminal activities, such as unauthorised access, disclosure and modification. *Implications for internal auditors*: the internal audit process must ensure the validity of software licences are relevant and updates are installed timely according to the manufacturer's recommendation.

CONCLUSIONS

This update has provided further references to support the implementation of the medical laboratory quality management system, particularly in Subclauses 4.6, 5.1, 5.2, 5.3 and 5.10 of ISO 15189:2012. The implementation of the PCDP stage of ISO 15189:2012 has the added complexity of having majority of CRs from Clause 5 of ISO 15189:2012; therefore, appropriate

level of attention with relevant resources are required to monitor the operational status of technical parameters. These findings are relevant to both implementers and internal auditors. In sum, ensuring reasonably practicable implementation of the PCDP stage of ISO 15189:2012 can ensure the relevant processes are controlled according to specifications of the organisation.

ACKNOWLEDGMENTS

The authors would like to thank Yuki Ohkawa, Medical Technologist, Kanazawa Medical University Hospital, Uchinada, Ishikawa, Japan, and Hideo Sakamoto, PhD, Department Chair and Professor, Department of Medical Technology, Faculty of Health Sciences, Kobe Tokiwa University, Kobe, Hyogo, Japan, for reading the manuscript and suggesting substantial improvements.

This article includes the studies and findings of the research supported by King Saud Medical City, under support order number H0QI-21-Oct19-02.

AUTHOR INFORMATION

Dennis Mok, Chartered MCIPD CSci MIBMS MIHRM(HK) CMgr FCMI FIML FNZIMLS CPHR, L&D Consultant[1]
Sharfuddin Chowdhury, MBBS MMed FCS(SA) FACS, Director of Trauma Center[2]

[1]Medical Management Consulting, Birkdale, Queensland, Australia
[2]King Saud Medical City, Riyadh, Riyadh, Saudi Arabia

Correspondence: Sharfuddin Chowdbury.
Email: s.chowdhury@ksmc.med.sa

REFERENCES

1. International Organization for Standardization. Medical laboratories — Requirements for quality and competence. 3rd edn. ISO 15189:2012. International Organization for Standardization, Geneva, 2014.
2. de Colle S. International Organization for Standardization (ISO). In: Kolb RW, ed. The SAGE encyclopedia of business ethics and society. 2nd edn. Vol. 4. SAGE Publications, Thousand Oaks, 2018: 1908-1910.
3. Organisation for Economic Co-operation and Development. International regulatory co-operation: the role of international organisations in fostering better rules of globalisation. OECD Publishing, Paris, 2016.
4. Özkan FZ, Pakdil F, Altuğ T. Accreditation of conformity assessment activities. In: Dahlgaard-Park SM, ed. The SAGE encyclopedia of quality and the service economy. Vol. 1. SAGE Publications, Thousand Oaks, 2015: 1-5.
5. Mok D. ISO 15189:2012 implementation checklists for conformity assessment by accreditation bodies: a comparative analysis. *N Z J Med Lab Sci* 2017; 71: 84-99.
6. Mok D, Chowdhury S. Document review checklists for ISO 15189:2012 internal auditing: an applied tool for medical laboratories. *Aust J Med Sci* 2016; 37: 48-55.
7. Mok D, Ang E. ISO 15189:2012 implementation: an update of related international standards and guidance documents for medical laboratory quality management. *N Z J Med Lab Sci* 2016; 70: 42-66.
8. Mok D, Chowdhury S. The strategic managment stage of ISO 15189:2012 management system standard: an implementation update. *N Z J Med Lab Sci* 2019; 73: 111-115.
9. Union of International Associations (Editors). *Yearbook of International Organizations 2019 - 2020: Guide to Global Civil Society Networks*. 56th edn. Vol. 1, Organization descriptions and cross-references, Koninklijke Brill, Leiden, 2019: 2894 pp.
10. International Organization for Standardization, International Electrotechnical Commission. Products and related services — Information for consumers. 3rd edn. ISO/IEC Guide 14:2018. International Organization for Standardization, Geneva, 2018.
11. Sadiku MN, Momoh OD. Firewalls. In: Warf B, ed. The SAGE encyclopedia of the internet. Vol. 2. SAGE Publications, Thousand Oaks, 2018: 380-382.

Supplementary material for this article is available at www.nzimls.org.nz/journal

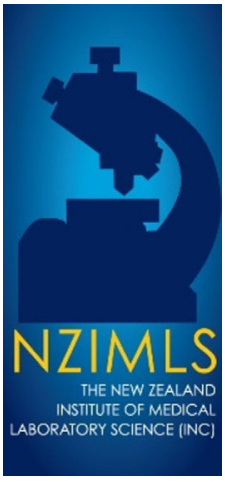
NZIMLS Journal Prize

The NZIMLS Council has approved an annual Journal prize to the value of NZ$300 for the best peer-reviewed article published by NZIMLS members in the Journal during the calendar year. The article can be a review article, original article, case study, research letter or technical communication. Excluded are Fellowship dissertations.

Many studies are presented at the Annual Scientific Meeting, SIG meetings, and the North and South Island Seminars, yet are rarely submitted to the Journal for wider dissemination to the profession. Consider submitting your presentation to the Journal. If accepted, you are in consideration for the NZIMLS Journal Prize and will also earn you valuable CPD points.

Please contact the Editor or any Editorial Board Member for advice and help. Contact details are on the NZIMLS web site (www.nzimls.org.nz) as are instructions to authors.

All articles published during the calendar year (April, August and November issues) will be considered. The Editor, Deputy Editor and the President of the NZIMLS, who themselves are ineligible, will judge all eligible articles in December. Their decision will be final and no correspondence will be entered into.



ORIGINAL ARTICLE

Apoptosis, dendritic cells and their ratio in the peripheral blood of patients with systemic lupus erythematosus

Douaa Sayed, Sonya M Rashad, Essam A Abbada, Ali Sobhy, Mohamed Allam and Eman NasrEldin

ABSTRACT

Aims: The aim of this study was to determine the populations of plasmacytoid and myeloid dentritic cells and to assess apoptosis and the apoptotic cell: peripheral blood dendritic cell ratio of systemic lupus erythematosis (SLE) patients.

Methods: Flow cytometric examinations of apoptosis, dendritic cells and their maturation for 29 SLE patients and 20 healthy controls were performed and apoptotic cell:dendritic cell ratios were measured.

Results: There was a significant decrease of inactivated and activated myeloid dendritic cell percentages and a non-significant increase of inactivated and activated plasmacytoid dendritic cells. There was a significanct increase of early apoptosis percentage, but no significanct difference of late apoptosis percentage. The latter showed a significance correlation between both inactivated and activated myeloid dendritic cells. There was a significant increase of early apoptosis to whole myeloid dendritic cell ratio.

Conclusions: Inactivated and activated myeloid dendritic cell percentages are decreased in SLE which might be the reason for incomplete removal of apoptotic cells and increase in the early apoptosis/whole myeloid dendritic cell ratio. This clarifies the expansion of apoptosis percentage in SLE patients and might be a vital factor in SLE pathogenesis.

Key words: dendritic cells, systemic lupus erythematosus, apoptosis.

N Z J Med Lab Sci 2020; 74: 107-114.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an immune system illness characterised by the creation of an enormous number of autoantibodies, with differing specificities as a consequence of abnormal activation of autoreactive B- and T-helper lymphocytes. In spite of the fact that the pathogenesis of the disease is to a great extent obscure, both hereditary and natural factors are involved (1).

Dendritic cells are bone marrow- inferred antigen presenting cells that have an essential part in the induction of immunity and upkeep of tolerance against self antigens. Dendritic cells play a key role in regulating immune responses, either by priming naive T-cells or tolerising autoreactive T-cells. T-cell priming necessitates mature dendritric cells whilst immature dendritic cells can motivate T-cell tolerance. Therefore, dysregulation of dendritic cell maturation to chronic dendritic cell activation may divert self-antigen presentation from tolerance to autoimmunity (2).

There are two unique kinds of dendritic cell populations that have been distinguished in blood and lymphoid organs. Both types are identified from other lymphoid and myeloid cells by their high expression levels of HLA-DR and also lack of lineage marker (CD3, CD19, CD14 and CD16) expression. $CD123^{-}$ and $CD11c^{+}$ dendritic cells. Myeloid dendritic cells differ considerably from $CD123^{+}$ and $CD11c^{-}$ plasmacytoid dendritic cells in their maturation requirements, cytokine production and functional capacities (3).

SLE patients are characterised by expanded number of the plasmacytoid dendritic cells in the peripheral blood and low number of myeloid dendritic cells. Plasmacytoid dendritic cells are constantly actuated to produce type I interferon, prompting enactment and maturation of myeloid dendritic cells that animate autoreactive T-cells, plasmacytoid dendritic cell - derived type 1 interferon, together with interleukin-6 (IL6) advance the differentiation of autoreactive B cells into autoantibody forming plasma cells (4).

Apoptotic cells are the source of a large portion of the antigens towards which the autoimmune response is focused in SLE. When denditric cells acquire apoptotic cell fragments, self-antigens are displayed in a way that leads to the inactivation of possible autoreactive T-cells through deletion, anergy, or development of regulatory cells. In this manner, apoptotic cell clearance and the introduction of related self-antigens have an imperative part in the conservation of immune tolerance. This mechanism, possibly influenced by many factors like the apoptotic cell: dendritic cell ratio, the type of surface receptors responsible for the internalization of apoptotic cell fragments, the denditric cells maturation state and other abnormalities intrinsic to denditric cells (5). Expanded apoptosis and weakened phagocytic clearance of apoptotic cells could assume vital parts in the breakdown of self-tolerance in SLE (6).

The aim of our study was to determine the populations of plasmacytoid and myeloid denditric cells and to assess the apoptosis and the apoptotic cell:denditric cell ratio in the peripheral blood of SLE patients.

METHODS

Patients

This study was conducted on randomly selected SLE patients (28 female, 1 male) with a mean age of 27.1 ± 7.46 years. All patients fulfilled the American Colleges of Rheumatology criteria for the classification of SLE. The control group comprised 20 healthy volunteers (15 female, 5 male) with a mean age of 26.6 ± 4.1 years. Written informed consent was obtained from all subjects and the study was approved by the Ethical Committee of the Faculty of Medicine, Assuit University, Egypt. All patients were submitted to a full history taking, clinical examination and laboratory investigations, while the control subjects were submitted to laboratory investigations only. The clinical investigations are categorised in Table 1.

Systemic lupus erythematosus disease activity index

This index incorporates 24 items, 16 clinical and 8 laboratory, and manifestations were recorded if present in the preceeding 10 days, paying little heed to seriousness or whether it was enhanced or weakened (7).

Specimen collection

10 ml of venous blood was obtained from all subjects by venipunctures and divided into three portions as follows: 2.0 ml was added to EDTA tubes for estimation of complete blood count and flow cytometric investigation; 1.6 ml was added to 0.4 ml of Na citrate for estimation of erythrocyte sedimentation rate (ESR), and the rest was allowed to clot at room temperature and then centrifuged at 4000 rpm. The separated serum was utilised for estimation of liver and kidney function tests, C reactive protein (CRP), rheumatoid factor (RF), antinuclear antibody (ANA) and anti double strand DNA (dsDNA). Urine analysis was performed for the presence of proteins and types of casts. 24 hour urine was collected for creatinine clearance and 24 hours protein.

Monoclonal antibodies

By immunostaining utilising fluorescein-isothiocyanate (FITC) conjugated mouse monoclonal antibodies (mAb) against CD3 (Beckman Coulter, USA), annexin v (IQ Products, Netherlands), CD14 and CD11c (Bioscience, Sandiego, Canada), phycoerythrin conjugated propidium iodide, CD19 (IQ Products, Netherlands), CD86 and CD123 (BD Bioscience, USA), HLA-DR peridinium-chlorophyll-protein compound (BD Bioscience, USA) and the corresponding mouse isotype controls (all mAbs from BD PharMingen, SanDiego, CA). mAbs were used at concentrations titrated for optimal staining. Three colour staining of mAbs were carried out using a standard protocol as per the following panel:

CD3/CD19/HLA-DR - CD11c/ CD123/ HLA-DR
CD14/ CD86/ HLA-DR - Annexin V/PI

50µl of whole blood was incubated for 20 min at 4°C in the dark with 10 µl of the combined antibodies. After incubation, the cells were washed with phosphate buffered saline (PBS), followed by lysis of erythrocytes [BD FACS lysing solution (10X)], after a second wash the cell pellet was resuspended in 50µl of PBS.

Flow cytometric analysis of dendritic cells and their maturation

Flow cytometry was performed with a FACS Calibur system (BD, San Jose, CA). All data were investigated and displayed with the CELL QUEST software program (BD, San Jose, CA), 80,000 - 100,000 events were acquired. Wide gate around all mononuclear cell regions was drawn to include all dendritic cells (R1). This gate excluded debris, dead cells, platelets aggregate and myeloid cells. Gated cells were reanalysed for expression of CD3, CD19, CD14, CD3/HLA-DR and CD14/ HLA-DR to identify T-lymphocytes, B-lymphocytes, monocytes, activated lymphocytes and activated monocytes (Figures 1A, 1B, 1C, 1D, 1E and 1F). Gated cells were re-analysed for expressions of CD11c and CD123 with or without HLA-DR expression to identify different dendritic cell types (Figures 1G, 1H and 1I). In addition, gated cells were re-analysed for expression of HLR-DR and CD86 to identify antigen presenting cell maturation and activation (Figure 2B).

All expressions were estimated by the level of (percentage) positive cells. Cut-off of positivity was resolved by isotypic control. Region (R2) was drawn to include all HLA-DR$^+$ cells on dot plot showing HLA-DR versus forward light scatter (Figure 2D). Cells in the R2 region were re-analysed for expression of CD86 and its percentage. We noticed that the expression of HLA-DR and CD86 varied in intensity from case to case, therefore histograms were used to illustrate mean fluorescence intensity for these markers (Figures 2C and 2E).

Flow cytometric analysis for apoptosis

Cells in region R1 were re-analysed for annexin V only expression and expression of both annexin V and PI to distinguish early and late apoptosis, respectively (Figure 2F).

Calculation of apoptosis and inactivated and activated myeloid dendritic cells ratios

The ratios between percentages of apoptotic cells (early or late) and myeloid dendritic cells (inactivated or activated) for SLE patients and healthy controls were ascertained as follows:

- Early apoptosis: Annexin V$^+$/ inactivated myeloid denditric cells (CD11c$^+$ CD123$^-$ HLA-DR$^-$).
- Early apoptosis: Annexin V$^+$/activated myeloid denditric cells (CD11c$^+$ CD123$^-$ HLA-DR$^+$).
- Late apoptosis: Annexin V$^+$ PI$^+$/inactivated myeloid denditric cells (CD11c$^+$ CD123$^-$ HLA-DR$^-$).
- Late apoptosis: Annexin V$^+$ PI+/activated myeloid denditric cells (CD11c$^+$ CD123$^-$ HLA-DR$^+$).

RESULTS

Clinical information of the SLE patients is presented in Table 1 while haematological, biochemical and immunological test results of patients and controls are presented in Table 2.

Immunophenotyping of T lymphocytes, B lymphocytes and monocytes cells

There was no significance difference between percentage of gated cells in SLE patients and normal controls. There was a significance increase of T-lymphocytes (CD3$^+$) and B-lymphocytes (CD19$^+$) percentage in SLE patients, compared to controls. As regards activated T-lymphocytes (CD3$^+$ HLA-DR$^+$) percentage, there was no significance difference between SLE patients and normal controls.

There was no significant difference in non-activated (CD14$^+$ HLA-DR$^-$) and activated monocytes percentages (CD14$^+$ HLA-DR$^+$) between SLE patients and normal controls (Table 3). There was a significance decrease of inactivated and activated myeloid dendritic cell percentages in SLE patients compared to controls, but there was an insignificant increase of inactivated and activated plasmacytoid dendritic cells (Table 3, Figure 3).

Antigen presenting cells immunophenotyping and HLA-DR expression

We found a significance decrease of HLA-DR$^+$ cell percentage, HLA-DR (MFI) and (CD86$^+$ HLA-DR$^+$) cell percentage in SLE patients compared to control subjects. There was a significant increase in CD86$^+$ HLA-DR$^-$ cell percentage but no difference of the CD86 MFI was found between SLE patients and normal controls (Table 4).

Apoptosis

Early apoptosis percentage (Annexin V$^+$) increased significantly but there was no significance difference of late apoptosis percentage (Annexin V$^+$ PI$^+$) between SLE patients and normal controls (Table 5, Figure 4).

Correlations

There were significant correlations between both inactivated and activated myeloid denditric cells and late apoptosis (Figure 5). No significant correlation was found between inactivated and activated plasmacytoid denditric cells and early and late apoptosis. There were negative correlations between both activated plasmacytoid denditric cells and activated myeloid denditric cells and T-lymphocytes (r=-0.391; p=0.03 and r=-0.346; p=0.066 respectively). There was a negative fair correlation between plasmacytoid denditric cells (inactivated and activated) and SLEDAI, although statistically insignificant (r=-0.321, p=0.226 and r=-0.318, p=0.271 respectively). There was a significant correlation between CD86$^+$ HLA-DR$^-$ cells percentage and SLEDAI (r=0.375, p=0.045) while there was a negative significant correlation between CD86$^+$ HLA-DR$^+$ cells percentage and SLEDAI (r=-0.409; p=0.028). There was a fair but non-significant correlation between CD86 MFI and SLEDAI (r=0.343; p=0.065).

Ratio between apoptosis and myeloid denditric cells

There was a significant increase of the early apoptosis/ inactivated myeloid denditric cell ratio but there were insignificant increases in the ratio of early apoptosis/activated myeloid denditric cells, late apoptosis/inactivated myeloid denditric cells and late apoptosis/activated myeloid denditric cells in SLE patients (Table 6).

Table 1. Clinical data of SLE patients (n=29).

Variable	Descriptive
Disease duration (months)	49.65 ± 37.63
Fever	
- Low grade	2 (6.9%)
- Moderate grade	6 (20.7%)
- High grade	14 (48.3%)
Fatigue	16 (55.2%)
Alopecia	22 (75.9%)
Myalgia, Myositis	6 (20.7%)
Arthralgia	24 (82.8%)
Arthritis	20 (69.0%)
Oral ulcers	20 (69.0%)
Malar rash	15 (51.7%)
Photosensitivity	19 (65.5%)
Skin rash	11 (37.9%)
Chest manifestation	11 (37.9%)
Cardiac manifestation	10 (34.5%)
CNS manifestation	8 (27.6%)
GIT manifestation	12 (41.4%)
Activity	16 (55.2%)

Quantitative variables expressed as mean ± SD. Qualitative variables expressed as number of positive cases (%).
SLE: systemic lupus erythematosus; CNS: central nervous system; GIT: gastrointestinal tract.

Table 2. Laboratory parameters in SLE patients (n=29) and normal controls (n=20).

Parameter	SLE patients	Normal controls	p
ESR (mm/hour)	69.7 ± 39.1	8.70 ± 2.53	< 0.000
Hemoglobin (g/L)	95.7 ± 18.1	121.6 ± 14.2	<0.000
TLC (x10^9/L)	6.22 ± 3.83	6.01 ± 2.00	0.824
Platelets count (x10^9/L)	326.8 ± 173.5	225.6 ± 62.1	<0.005
ALT (U/L)	21.31 ± 6.83	23.55 ± 5.54	0.23
AST (U/L)	21.06 ± 5.16	23.05 ± 3.50	0.142
Albumin (g/L)	32.6 ± 5.5	43.6 ± 2.4	<0.000
Uric acid (g/L)	262.4 ± 41.7	245.7 ± 50.6	0.205
Creatinine clearance (ml/min/1.73m2)	80.2 ± 21.7	92.2 ± 9.0	0.024
Urine total protein (g/24h)	1.09 ± 0.75	0.04 ± 0.03	<0.01
ANA	6.17 ± 2.42	0.26 ± 0.19	<0.000
Anti ds DNA (IU/mL)	66.68 ± 36.43	6.75 ± 3.25	<0.000

Variables expressed as mean ± SD
SLE: systemic lupus erythematosus; ESR: erythrocyte sedimentation rate; TLC: total leucocytic count; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ANA: antinuclear antibody; Anti ds DNA: anti double strand deoxyribonucleic acid.

Table 3. Immunophenotyping of different cell types in SLE patients (n=29) and normal controls (n=20).

Cell type	SLE patients	Normal controls	p
Gated cells (%)	9.65 ± 4.70	9.60 ± 4.54	0.980
$CD3^+$ (%)	23.84 ± 17.60	12.56 ± 10.42	<0.013
$CD19^+$ (%)	10.56 ± 3.51	4.07 ± 3.52	<0.04
$CD3^+$ $HLA\text{-}DR^+$ (%)	4.90 ± 1.25	4.88 ± 2.31	0.987
$CD14^+$ $HLA\text{-}DR^-$ (%)	5.65 ± 2.07	1.79 ± 0.77	0.143
$CD11c^+$ $CD123^-$ $HLA\text{-}DR^-$ (%)	20.09 ± 4.09	45.21 ± 3.20	<0.000
$CD123^+$ $CD11c^-$ $HLA\text{-}DR^-$ (%)	4.64 ± 0.70	3.15 ± 0.43	0.162
$CD11c^+$ $CD123^-$ $HLA\text{-}DR^+$ (%)	26.28 ± 6.94	52.81 ± 18.30	<0.000
$CD123^+$ $CD11c^-HLA\text{-}DR^+$ (%)	16.14 ± 2.96	10.77 ± 7.88	0.171

Results expressed as mean ± SD.
SLE: systemic lupus erythematosus; CD: cluster of differentiation; HLA: human leukocyte antigen

Table 4. Immunophenotyping of APCs maturation and HLA DR expression in SLE patients (n=29) and normal controls (n=20).

Variable	SLE patients	Normal controls	p
HLA-DR^{+} (%)	50.02 ± 26.64	68.14 ± 18.17	0.01
HLA-DR MFI	433.00 ± 115.48	768.78 ± 98.43	<0.04
CD86^{+} HLA-DR^{-} (%)	21.08 ± 3.69	3.38 ± 1.03	<0.000
CD86^{+} HLA-DR^{+} (%)	50.24 ± 17.18	108.86 ± 41.12	<0.000
CD86 MFI	98.37 ± 40.44	110.15 ± 40.22	0.320

Variables expressed as mean ± SD.
SLE: systemic lupus erythematosus; CD: cluster of differentiation; HLA: human leukocyte antigen; MFI: mean fluorescence intensity.

Table 5. Comparison of early and late apoptosis in SLE patients (n=29) and normal controls (n=20).

Variable	SLE patients	Normal controls	p
Early apoptosis: Annexin V^{+} (%)	8.26 ± 7.78	3.29 ± 1.33	<0.02
Late apoptosis: Annexin V^{+} PI^{+} (%)	2.33 ± 2.72	3.90 ± 0.914	0.113

Results expressed as mean ± SD.
SLE: systemic lupus erythematosus.

Table 6. Ratio between early and late apoptosis and inactivated and activated myeloid denditric cells in SLE patients and normal controls.

Variables	SLE patients	Normal controls	p
Early apoptosis/inactivated mDCs	1.2215 ± 0.37 7	0.0887 ± 0.036	<.017
Early apoptosis/activated mDCs	0.344 ± 0.137	0.0948 ± 0.022	0.141
Late apoptosis/inactivated mDCs	1.2274 ± 0.557	0.0951 ± 0 .05	0.10
Late apoptosis /activated mDCs	0.3487 ± 0.1543	0.0895 ± 0.02411	0.174

SLE: systemic lupus erythematosus; mDCs: myeloid dendritic cells; pDCs: plasmacytoid dendritic cells.

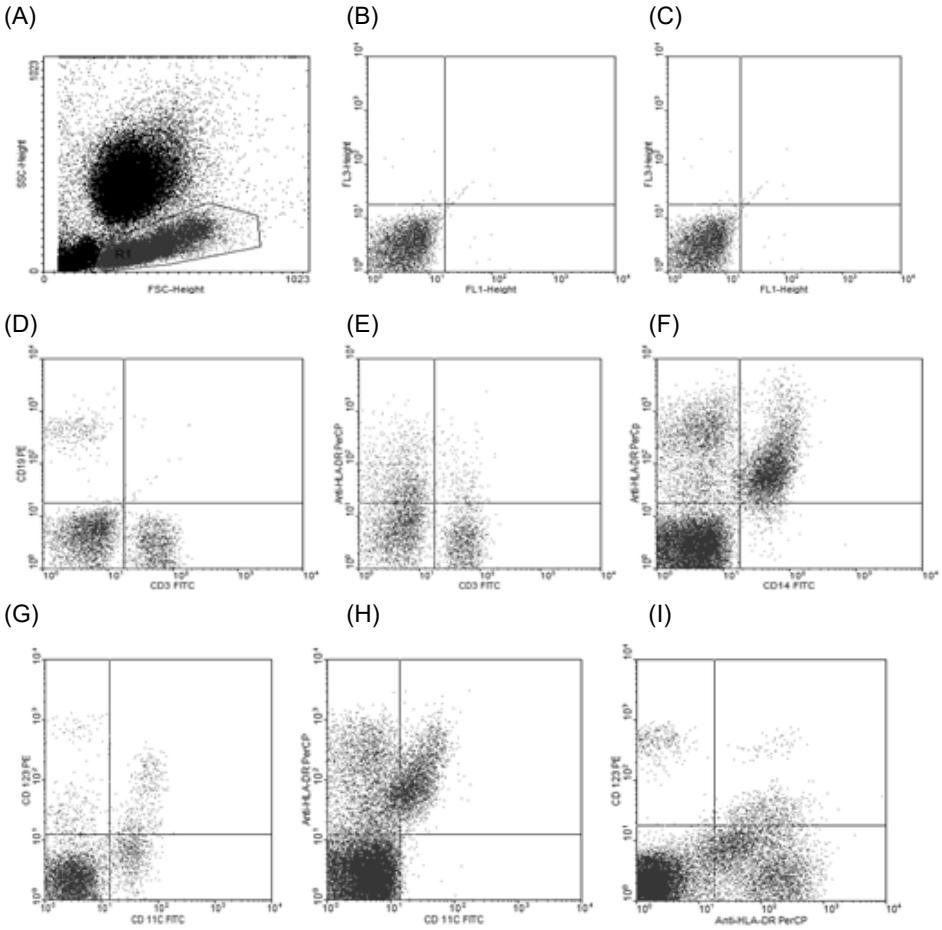


Figure 1.
(A): Dot plot of FSC vs. SSC showing a distinct population (R1) of mononuclear cells.
(B): dot plot of FL1 vs. FL2 gated on R1 showing clear isotypic control

(C): Dot plot of FL1 vs. FL3 gated on R1 showing clear isotypic control.
(D): Dot plot of CD3 FITC vs. CD19 PE gated on R1 showing two population groups, the first is of T-lymphocytes and the second is of B-lymphocytes.
(E): Dot plot of CD3 FITC vs. HLA-DR Per CP gated on R1 showing three population groups, the first is of T-lymphocytes that express CD3, the second is of APCs that express HLA-DR and the third population co-expresses two markers representing activated lymphocytes.
(F): Dot plot of CD14 FITC vs. HLA-DR Per CP gated on R1 showing two population groups, the first is of activated monocytes that co-express both markers and the second is of other APCs that express HLA-DR.
(G): Dot plot of CD11c FITC vs. CD123 PE gated on R1 showing two population groups, the first is of myeloid denditric cells that express CD11c and the second of plasmacytoid denditric cells that express CD123.
(H): Dot plot of CD11c FITC vs. HLA-DR Per CP gated on R1 showing that activated myeloid denditric cells co-express CD11c and HLA-DR.
(I): Dot plot of CD123PE vs. HLA-DR Per CP gated on R1 showing that activated plasmacytoid denditric cells co-express CD123 and HLA-DR.

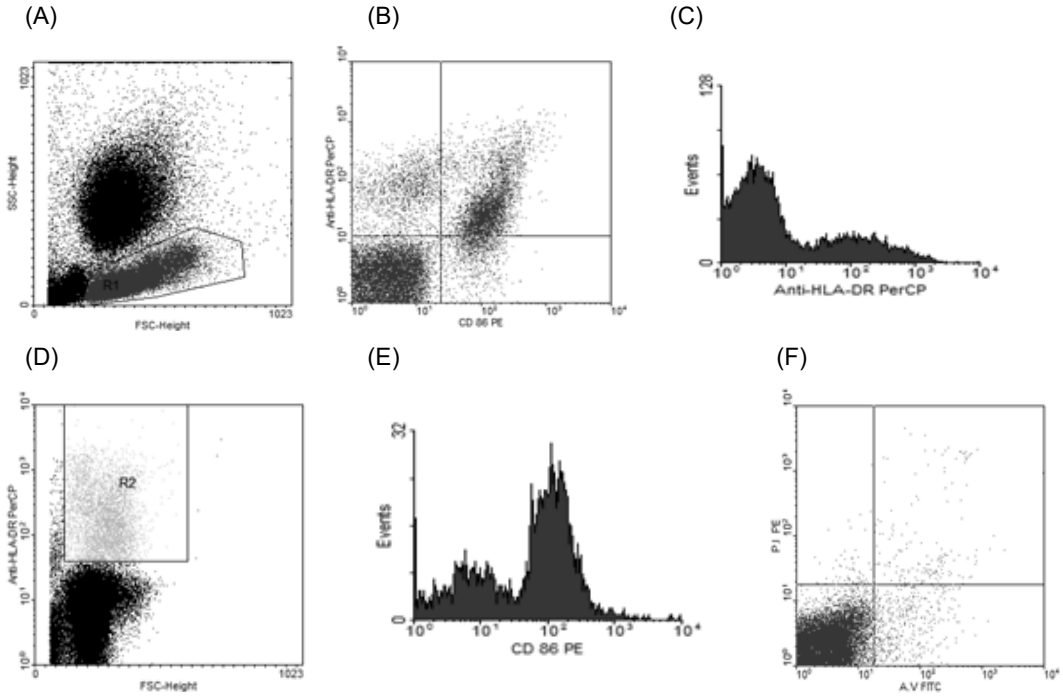


Figure 2.
(A): Dot plot of FSC vs. SSC showing a distinct population (R1) of mononuclear cells.
(B): Dot blot of CD86 PE vs. HLA-DR Per CP gated on R1 showing two population groups, the first of which is of mature APCs co-express CD86 and HLA-DR and the second express HLA-DR represent other APCs.
(C): Histogram showing mean fluorescence intensity of HLA-DR gated on R1.
(D): Dot plot of HLA-DR vs FSC showing distinct population of APCs (R2).
(E): Histogram showing mean fluorescence intensity of CD86 gated on R2.
(F): Dot plot of annexin V FITC vs. propidium iodide PE showing few cells expressing annexin V which represents early apoptosis and few cells with co-expression of annexin V and propidium iodide which represent late apoptosis.

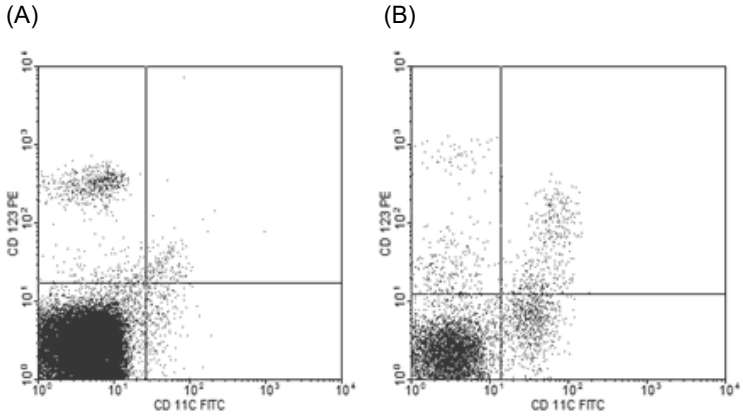


Figure 3.
(A): Dot plot of CD11c FITC vs. CD123 PE showing increased percentage of plasmacytoid denditric cells that express CD123 and decreased percentage of myeloid denditric cells that express CD11c in SLE patients.
(B): Dot plot of CD11c FITC vs. CD123 PE showing decreased percentage of plasmacytoid denditric cells that express CD123 and increased percentage of myeloid denditric cells that express CD11c in normal controls.

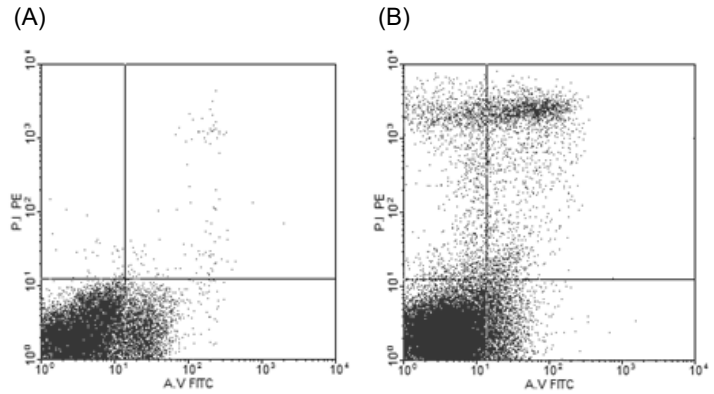


Figure 4.
(A): Dot plot of annexin V FITC vs. propidium iodide PE showing increase of early apoptosis percentage (Annexin v^+) in SLE patients.
(B): Dot plot of annexin V FITC vs. propidium iodide PE showing increase of late apoptosis percentage (Annexin V^+ propedium $iodide^+$) in SLE patients.

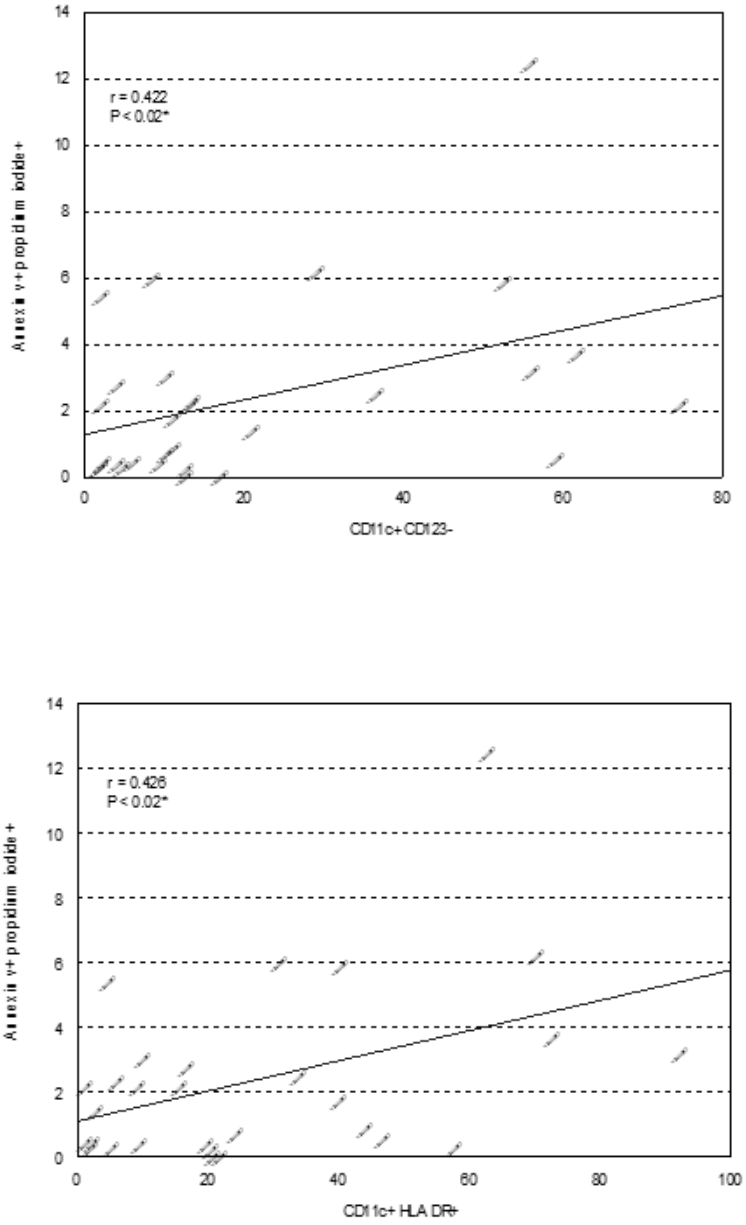


Figure 5. Correlations between inactivated myeloid denditric cells and late apoptosis and between activated myeloid denditric cells and late apoptosis.

DISCUSSION

In our study the level of myeloid denditric cell in peripheral blood diminished in SLE patients compared to healthy controls. This result is consistent with most of the published literature. Schcincker *et al.* reported several possible explanations for the reduced proportions of myeloid denditric cells subsets in SLE (9). Firstly: immature denditric cell progenitors might be reduced, or their differentiation toward myeloid denditric cell might be disturbed in SLE. Secondly: denditric cells might turn over more quickly in SLE. Lastly: activated denditric cells might be specifically selected for recruitement to inflammation sites. We support the hypothesis of disturbance and unsettling influence in the differentiation into myeloid denditric cell as the reduction was in both inactivated and activated myeloid denditric cell subtypes, while on the other hand plasmacytoid denditric cells increased. The increase of peripheral blood plasmacytoid denditric cell observed in our study is consistent with previous studies (10,11). However, other studies have reported that a percentage of plasmacytoid denditric cell are decreased in SLE patients (2,9,12). These conflicting data may be due to different methods used in plasmacytoid denditric cell detection.

T-cells have a pivotal part in patients with SLE in respect to disease progression and pathology. T-cells advance inflammation by emitting cytokines and activating denditric and B-cells in SLE. In our study, T-lymphocyte percentages were increased in SLE patients, compared to healthy controls, without an increase in activated T-lymphocytes that may be due to compensatory mechanisms to defects in its function. This may indicate a decline in processing and in presentation of antigens by T-cells in SLE. This expansion in T-lymphocytes likewise might be a compensatory mechanism to the defects occurring in the function of denditric cells, It is suggested that this is due to the negative correlations between T-cells and activated myeloid denditric cells and plasmacytoid denditric cells as observed in our study. In our study, during apoptotic cell and denditric cell maturation in SLE disease, HLA-DR expression and HLA-DR MFI were decreased, compared to healthy controls. This result is consistent with previous studies (10,11,13). Those studies reported that activated or mature myeloid denditric cells in SLE patients express low level of HLA-DR, compared to controls. Those studies suggested that deficiency in HLA-DR expression might be the cause of increased susceptibility of patients with SLE to various infections (13).

In contrast to our results, Ding *et al.* reported that myeloid denditric cells in SLE patients express higher levels of HLA-DR (3), Decker *et al.* found that they express normal levels of HLA-DR (1), while Crispin and Alcocer-Varela clarified the different results for the previous two studies as those did not use patient-derived sera during the differentiation process (14). Therefore, differentiation and maturation of dendritic cells from SLE patients appear to be abnormal.

A new small population of cells which expresses CD86 without expression of HLA-DR was recognised in our study and was increased in SLE patients compared to normal control subjects. The percentage of these cells ($CD86^+$ $HLA\text{-}DR^-$) and CD86 MFI show fair correlations with SLEDAI. These cells may be the cause as other studies have reported that CD86 expression increases in denditric cells in SLE patients (1,3,10,11). In contrast, earlier studies by Schcincker *et al.* reported that in SLE, dendritric cells express low levels of CD86 (9), while Koler *et al.* reported that in SLE, denditric cells express normal levels of CD86 (13). The explanation for this discrepancy may be that later studies detected CD86 expression on $HLA\text{-}DR^+$ cells. In our study ($CD86^+$ $HLA\text{-}DR^+$) cell percentage was also decreased in SLE patients.

A significant characteristic of myeloid denditric cells in SLE is the capability to pick up both apoptotic and necrotic cell material and their presentation to T cells**.** In contrast to myeloid denditric cell, plasmacytoid denditric cells are unable to ingest uncomplexed apoptotic and necrotic material (15).

This might be the clarification of our correlation results with respect to the relation between both myeloid and plasmacytoid denditric cells and apoptosis.

Strong evidence indicates that insufficient clearance is probably a more important cause of the increased load of apoptotic material in SLE (16). In our study, early apoptosis was increased in SLE patients. This could be due to disabled clearance of apoptotic cells as a consequence of diminishing myeloid denditric cell percentage. We found no significant difference in late apoptosis percentage between SLE patients and healthy controls. The significant correlation between myeloid denditric cells to late apoptosis may be the cause of latency to stimulate myeloid denditric cells. Our results is consistent with previous studies (17-23) It is a result of defective phagocytic capacity of myeloid denditric cells and over-burden of apoptotic cells over myeloid denditric cells' ability to remove apoptotic cells. We thus analysed the ratio between apoptosis and myeloid denditric cells which revealed that the early apoptosis/whole myeloid denditric cell ratio was increased in SLE patients. To the best of our knowledge, no one has examined the ratio of apoptosis/ myeloid denditric cells in SLE patients before. These results explain the increase of apoptosis percentage in SLE patients and may be considered an important factor in the pathogenesis of SLE. We recommend a more larger study with follow up of cases to obtain more verifiable results.

In conclusion, inactivated and activated myeloid denditric cell percentages are decreased in SLE which might be the reason for their incomplete removal of apoptotic cells and increased early apoptosis percentage. Additionally, inactivated and activated plasmacytoid denditric cell percentages increase in SLE patients. Mature apoptotic cells, including denditric cells, had low expression of HLA-DR and another small population of cells expresses CD86 without HLA-DR expression was detected and was increased in SLE patients. This indicates that differentiation of denditric cells, and also its maturation, in SLE patients seem to be abnormal. Early apoptosis/whole myeloid denditric cell ratio was greater in patients with SLE. This clarifies the increase of apoptosis percentage in SLE patients and may be eminent part in SLE pathogenesis.

AUTHOR INFORMATION

Douaa Sayed, MD, Professor[1]
Sonya M Rashad, MD, Profesor[2]
Essam A Abbada, MD, Professor[2]
Ali Sobhy, MD, Assistant Professor[3]
Mohamed Allam, MD, Professor[3]
Eman NasrEldin, MD, Assistant Professor[4]

[1]Flow Cytometry Laboratory, Department of Clinical Pathology, South Egypt Cancer Institute, Assiut University, Egypt
[2]Department of Rheumatology and Rehabilitation, Faculty of Medicine, Assiut University, Egypt
[3]Department of Clinical Pathology, Faculty of Medicine, Al-Azhar University, Assiut, Egypt
[4]Department of Clinical Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt.

Correspondence: Eman NasrEldin
E-mail: emannasr2000@yahoo.com

REFERENCES

1. Decker P, Kötter I, Klein R, Berner B, Rammensee HG. Monocyte-derived dendritic cells over-express CD86 in patients with systemic lupus erythematosus. *Rheumatology (Oxford)* 2006; 45: 1087–1095.
2. Migita K, Miyashita T, Maeda Y, Kimura H, Nakamura M, Yatsuhashi H, et al. Reduced blood BDCA-2+ (lymphoid) and CD11c+ (myeloid) dendritic cells in systemic lupus erythematosus. *Clin Exp Immunol* 2005; 142: 84–91.

3. Ding D, Mehta H, McCune WJ, Kaplan MJ. Aberrant phenotype and function of myeloid dendritic cells in systemic lupus erythematosus. *J Immunol* 2006; 177: 5878-5889.
4. Lande R, Gilliet M. Plasmacytoid dendritic cells: key players in the initiation and regulation of immune responses. *Ann N Y Acad Sci* 2010; 1183: 89-103. Erratum in *Ann N Y Acad Sci* 2010; 1188: 232.
5. Stuart LM, Lucas M, Simpson C, Lamb J, Savill J, Lacy-Hulbert A. Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. *J Immunol* 2002; 168: 1627–1635.
6. Chen XW, Shen Y, Sun CY, Wu FX, Chen Y, Yang CD. Anti- class a scavenger receptor autoantibodies from systemic lupus erythematosus patients impair phagocytic clearance of apoptotic cells by macrophages in vitro. *Arthritis Res Ther* 2011; 31 ; 13(1): R9.
7. Yee CS, Isenberg DA, Prabu A, Sokoll K, Teh LS, Rahman A, et al. BILAG-2004 index captures systemic lupus erythematosus disease activity better than SLEDAI-2000. *Ann Rheum Dis* 2008; 67: 873–876.
8. Boersma HH, Kietselaer BL, Stolk LM, Bennaghmouch A, Hofstra L, Narula J, et al. Past, present, and future of annexin A5: from protein discovery to clinical applications. *J Nucl Med* 2005; 46: 2035-2050.
9. Scheinecker C, Zwölfer B, Köller M, Männer G, Smolen JS. Alterations of dendritic cells in systemic lupus erythematosus: phenotypic and functional deficiencies. *Arthritis Rheum* 2001; 44: 856–865.
10. Jin O, Kavikondala S, Sun L, Fu R, Mok MY, Chan A, et al. Systemic lupus erythematosus patients have increased number of circulating plasmacytoid dendritic cells, but decreased myeloid dendritic cells with deficient CD83 expression. *Lupus* 2008; 17: 654-662.
11. Nie YJ, Mok MY, Chan GC, Chan AW, Jin OU, Kavikondala S, Lie AK et al. Phenotypic and functional abnormalities of bone marrow-derived dendritic cells in systemic lupus erythematosus. *Arthritis Res Ther* 2010; 12 (3): R91.
12. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science* 2001; 294: 1540–1543.
13. Köller M, Zwölfer B, Steiner G, Smolen JS, Scheinecker C. Phenotypic and functional deficiencies of monocyte-derived dendritic cells in systemic lupus erythematosus (SLE) patients. *Int Immunol* 2004; 16: 1595–1604.
14. Crispin JC, Alcocer-Varela J. The role myeloid dendritic cells play in the pathogenesis of systemic lupus erythematosus. *Autoimmun Rev* 2007; 6: 450-456.
15. Fransen JH, van der Vlag J, Ruben J, Adema GJ, Berden JH, Hilbrands LB. The role of dendritic cells in the pathogenesis of systemic lupus erythematosus. *Arthritis Res Ther* 2010; 12(2): 207.
16. Munoz LE, van Bavel C, Franz S, Berden J, Herrmann M, van der Vlag J. Apoptosis in the pathogenesis of systemic lupus erythematosus. *Lupus* 2008; 17: 371-375.
17. Bengtsson AK, Ryan EJ, Giordano D, Magaletti DM, Clark EA. 17beta-estradiol (E2) modulates cytokine and chemokine expression in human monocyte-derived dendritic cells. *Blood* 2004;104: 1404-1410.
18. Robak E, Sysa-Jedrzejowska A, Robak T, Smolewski P. Peripheral blood lymphocyte apoptosis and circulating dendritic cells in patients with systemic lupus erythematosus: correlation with immunological status and disease-related symptoms. *Clin Rheumatol* 2006; 25: 225-233.
19. Dhir V, Singh AP, Aggarwal A, Naik S, Misra R. Increased T-lymphocyte apoptosis in lupus correlates with disease activity and may be responsible for reduced T-cell frequency: a cross-sectional and longitudinal study. *Lupus* 2009; 18: 785-791.
20. Midgley A, McLaren Z, Moots RJ, Edwards SW, Beresford MW. The role of neutrophil apoptosis in juvenile-onset systemic lupus erythematosus. *Arthritis Rheum* 2009 ; 60: 2390-2401.
21. Fransen JH, Hilbrands LB, Jacobs CW, Adema GJ, Berden JH, Van der Vlag J. Both early and late apoptotic blebs are taken up by DC and induce IL-6 production. *Autoimmunity* 2009 ; 42: 325-327.
22. Habib HM, Taher TE, Isenberg DA, Mageed RA. Enhanced propensity of T lymphocytes in patients with systemic lupus erythematosus to apoptosis in the presence of tumour necrosis factor alpha. *Scand J Rheumatol* 2009; 38: 112-120.
23. Fransen JH, Dieker JW, Hilbrands LB, Berden JH, van der Vlag J. Synchronized turbo apoptosis induced by cold-shock. *Apoptosis* 2011; 16: 86-93.

NZIMLS Research Grant

A Research Grant of up to $5,000.00 is available to applicants who:

- Have been a member of the New Zealand Institute of Medical Laboratory Science (Inc.) for a minimum period of two years
- Are currently employed in an IANZ accredited laboratory
- Have fully completed the Application Form (online)

Applicants are encouraged to supply a final written report to the NZIMLS Journal Editor (within 12 months of completion of the project) for consideration of publication in the Journal of Medical Laboratory Science.

The decision of the NZIMLS Council on the awarding of Grants is final, and no correspondence will be entered into.

Application for the NZIMLS Research Grant is to be completed in full on the forms available at:
https://www.nzimls.org.nz/research-grant.html

Forward your completed application to:
The Executive Officer
Fran van Til
NZIMLS, PO Box 505, Rangiora 7440
email: fran@nzimls.org.nz

ORIGINAL ARTICLE

Evaluating the clinical application of nested PCR in the diagnosis of fungal infections

Nasrin Sheikhi, Zahra Rafat, Saeed Zaker Boostan Abad, Sina Mirza Ahmadi, Reza Azizian and Masoud Hamidi

ABSTRACT

Background: Molecular techniques are appealing for the detection of human pathogens, but the detection of fungal pathogens is particularly challenging. Fungi have cell walls that impede the efficient lysis of organisms and liberation of DNA, which can lead to false-negative results. Conversely, some human pathogens are ubiquitous environmental saprophytes that can contaminate PCR reagents and cause false-positive results.

Aims and methods: In a cross-sectional study in Tehran, Iran from January 2017 to August 2018 we aimed to evaluate the incidence of fungal infections in 420 specimens from patients suspected of fungal disease and determined the clinical application of nested PCR for the diagnosis of fungal infections. Direct examination with 10% potassium hydroxide, culture on mycological media and nested polymerase chain reaction (PCR) by amplifying the conserved sequences of internal transcribed spacer 1 (ITS1) ribosomal DNA were performed in this study.

Results: 79 (18.8 %) out of 420 specimens were positive by direct examination, including 64 (81%) dermatophytes and 15 (19 %) Aspergillus spp. 52 (12.3%) had positive culture including 40 (76.9 %) dermatophytes and 12 (23.1) Aspergillus spp. 76/79 isolates were detected by nested-PCR (96.2%).

Conclusions: Our results showed that the incidence of fungal infections in the study population was relatively high and dermatophytes were the most common cause of fungal infections. Nested PCR had a high sensitivity and may be useful as a reliable screening method, especially in immunosuppressed and immunocompromised patients.

Keywords: Aspergillus, dermatophytes, direct smear, nested PCR, culture.

N Z J Med Lab Sci 2020; 74: 115-118.

INTRODUCTION

Fungal infections are a major cause of morbidity and mortality in recent decades. Even among those that are not serious, they can have certain harmful effects on patients' psychological, social and business functioning and can consume a considerable proportion of health care costs (1-3). Early diagnosis of fungal infections is essential for appropriate treatment (4). Saprophytes and common pathogenic fungi have been detected routinely by Gram and lactophenol cotton blue stains or with 10% KOH direct staining (4). For species determination of dermatophytes and other fungi, culture on Sabouraud dextrose and other mycological media are required. There are many problems in the traditional diagnosis with direct exam smear, culture and serology. These methods are time consuming and do not have critical sensitivity. Sensitivity of microscopy for diagnosis of fungal infection varies with the individual agent, the source and quality of the specimen and the skills and experience of the laboratorian (4). Cultures from clinical samples has the advantage of yielding the specific etiological agent, if positive. Moreover, culture allows for susceptibility testing. However, the use of culture for the diagnosis of fungal infections has significant limitations. It may take many days for results with several of filamentous fungi. For example, in disseminated candidiasis, while waiting for the result of blood culture one may miss ≥ 50% of patients with disseminated disease (5-7). It also typically takes 24–72 h for identification of *Candida* which is too long to initiate early treatment. Immunologic assays, such as Ag-Ab methods, are rapid but do not have sufficient specificity and accuracy and for immunosuppressed patients are not useful. In addition, diagnostic tests based on galactomannan (GM) antigen and glucan do not detect all fungal pathogens and have problems with specificity (8).

PCR as a conventional molecular method is used routinely throughout the world and has proper sensitivity and specificity for detecting infections in a timely manner. Nested-PCR exploits in two steps to increase sensitivity and specificity of conventional PCR and increases replication of the target gene (7). Due to the necessity of early diagnosis of fungal infections and choice of anti-fungal treatment as first step for hospitalised patients in ICU and other special care wards, nested PCR can be a rapid and reliable assay (8). In some cases, fungal infections have not been detected at an early stage and anti-fungal treatment was not started until the late phase of their disease. There is therefore an increased emphasis on the use of applicable, accurate and timely molecular methods for the diagnosis of fungal diseases. The aim of this study was to determine the clinical application of nested PCR for the diagnosis of fungal infections.

METHODS

Sampling

Samples were collected from Razi and Baghyatalah hospitals and the Massoud Clinical Laboratory in Tehran, Iran. Between January 2017 and August 2018 420 specimens were collected from the skin of the groin, axillary, sole of foot, face, head, back, hand, leg, fingernail, toenail and maxillary sinus. All samples were prepared by KOH 10% and were inoculated on Sabouraud dextrose agar (SDA, Merk), SDA with 5% chloramphenicol and cycloheximide in duplicate for dermatophyte and SDA with 5% chloramphenicol triplicate for mould isolation and incubated for 21 days at 30 °C. All isolates were subjected to nested-PCR technique. All samples with a positive result in the direct examination were subjected to the nested-PCR technique.

DNA extraction and nested-PCR

DNA was extracted by Probe NA (Qiagene Co.) and then purification and qualification of DNA checked on 0.8% agarose by amplifying the conserved sequences of internal transcribed spacer 1 (ITS1) ribosomal DNA. *Aspergillus* species was detected by nested-PCR using two pairs of primers; SP5-SP8 and ASP7-ASP1.- First step: SP5 (5'-GAT AAC GAA CGA GAC CTC GG-3'), SP8 (5'-TGC CAA CTC CCC TGA GCC AG-3'). Second step: ASP7 (5'-CCT GAG CCA GTC CGA AGG CC-3'), ASP1 (5'-CGG CCC TTA AAT AGC CCG GTC-3'). Dermatophyte species were detected by nested-PCR followed by two pairs of primers, CHS1-S-CHS1-R and JF2-JR2. First step: CHS1-S (5'-CAT CGA GTA CAT GTG CTC GC-3'), CHS1-R (5'-CTC GAG GTC AAA AGC ACG CC-3') Second step: JF2 (5'- GCA AAG AAG CCT GGA AGA AG-3') and JR2 (5'-GGA GAC CAT CTG TGA GAG TTG-3').

RESULTS

There were 79 positive direct smears, 50 from men and 29 from women with a combined age range of 11-87 years. Most infections were in 10 to 19-year-old males (Figure 1). Of the positive smears 3.5% of samples were *Aspergillus* spp. and 15.2% were dermatophytes. Hands were the most frequent anatomical site of the body that were infected, more so in men than women while the ear and axilla were the lowest frequent infected anatomical site of the body (Figure 2).

Of the 15 direct smears that were detected as *Aspergillus* spp., 12 (80%) had growth while all had positive PCR result (100%). Forty cases (62.5%) of dermatophytes that were detected by smear exam had growth while 96.8% had a positive PCR. Two samples that were detected by smear exam as dermatophytes, by PCR and culture methods were detected as *Aspergillus* spp.

Macroscopic and microscopic colony characteristics of isolates indicated that *T. mentagrophyte* (n=16, 40%) was the predominant dermatophytic species isolated followed by *T. rubrum* (n=*5*, 12.5%), *T. verrucosum* (n=*5*, 12. 5%), *T. tonsurans* (n=2, 5%) and E. *floccosum* (n=6, 15%). *Aspergillus flavus* (n=6, 50%) was the predominant *Aspergillus* species isolated followed by *Aspergillus niger* (n=2, 16.6%) and *Aspergillus fumigatus* (n=1, 8.3%). Three species (25%) were unknown.

There was a significant difference between fungal species in regard to different anatomical sites of the body (CI=0.95; P=0.000). *T. mentagrophyte* was the most common dermatophyte with infected hands as the most frequent infected anatomical site in both genders. *Aspergillus flavous* was the most frequent species that contaminated nails.

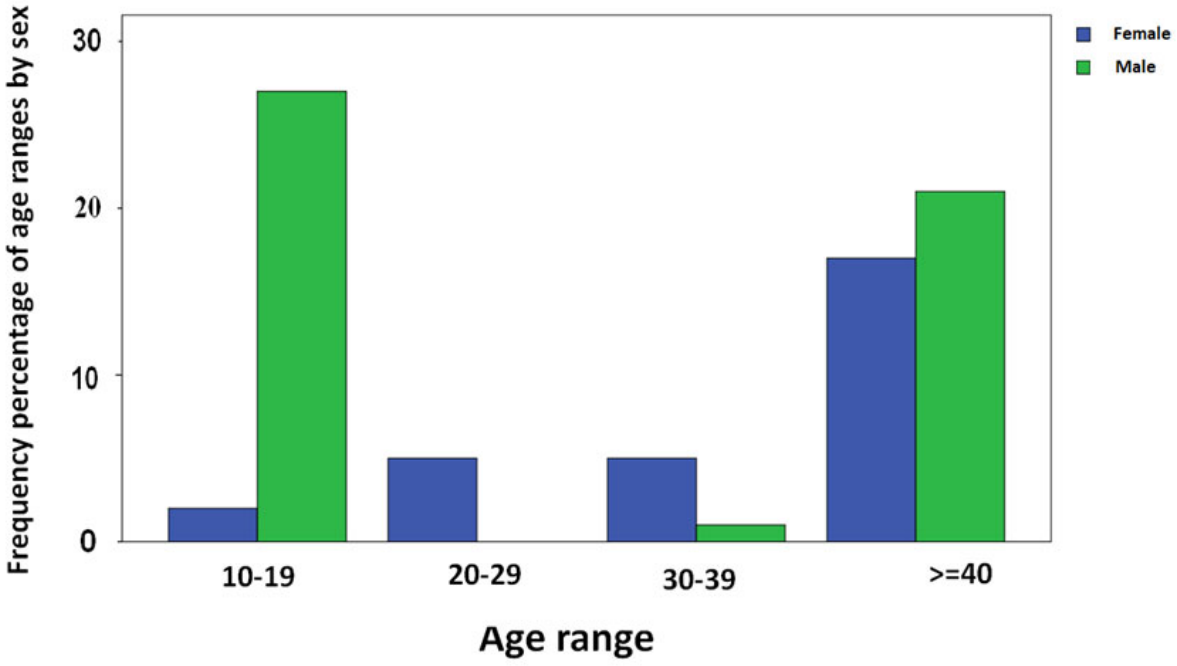


Figure 1. Prevalence of fungal infections by age and sex

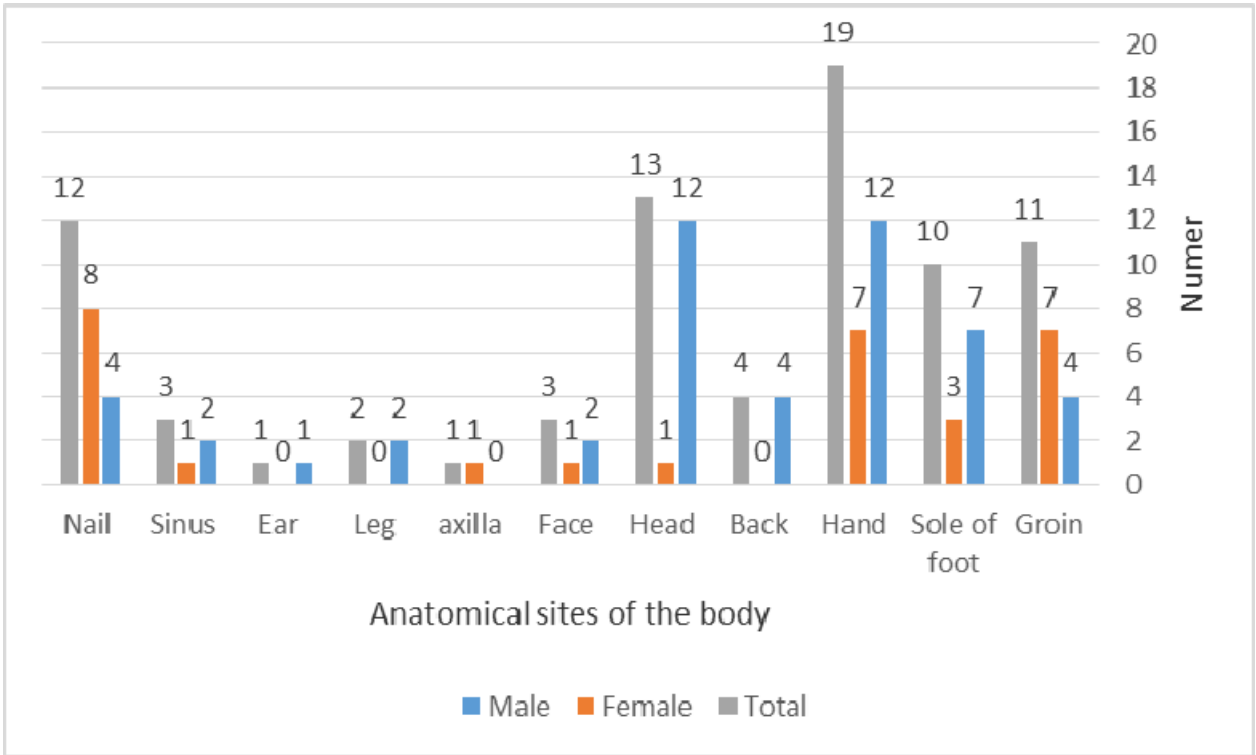


Figure 2. Frequency of infected anatomical sites of the body based on gender

DISCUSSION

There has been an enormous increase in the incidence and severity of fungal infections in the last decade due to the global HIV/AIDS epidemic (with patients that are susceptible to infections from fungi rarely seen, or never reported as a human pathogen) and advances in medical care and treatment that have led to increases in the number of opportunistic infections in patients who are immunocompromised or immunosuppressed. Diagnosis of fungal infections is challenging because current diagnostic methods lack sensitivity and specificity or take too long to yield a result to be clinically useful. Such limitations have consequences, delayed diagnosis leading to delayed treatment. Speed to diagnosis is a key factor in patient outcomes (8-11). The ideal test would detect infection early in the course of the disease, perhaps before the advent of symptoms. Early diagnosis would enable administration of antifungals at a time when treatment is most likely to be effective. Diagnosis of fungal infections has relied primarily on methods such as direct microscopic examination of clinical samples culture. One of the most important problems in the diagnosis of fungal infections with direct microscopic examination is the loss of senior mycology experts in the field. Furthermore, mycologists in the past needed to be able to identify 50 commonly encountered fungi, and 300 total fungi that were pathogenic for humans but the number of potential fungal pathogens is likely many times what is described in textbooks and will continue to grow as the severely immunosuppressed patient population continues to grow (12,13). Additionally, one of the most significant limitations of using culture for the diagnosis of fungal infections is that it may take many days for a result with several of the filamentous fungi.

The growth in the number of fungi that clinical mycologists must be able to identify has forced investigators to develop and apply new methods for fungal identification that go beyond classical phenotypic methods. In our study we evaluated the use of nested PCR as a molecular method in the diagnosis of fungal infections. However, nested PRC in comparison to direct examination and culture is expensive. In this study we aimed to determine the sensitivity of the nested PCR method in true-positive specimens in which the presence of fungal elements had already been confirmed by direct examination. Results indicate that nested PCR has a high sensitivity and specificity in fungal detection and may be considered as a gold standard for the diagnosis of fungal infections which can aid the clinician in initiating prompt and appropriate antifungal therapy, especially in severe immunosuppressed patients in whom an accurate diagnosis of the causative agent and timely treatment is critical. Our findings are consistent with the findings of Garg *et al*. (14). A previous study evaluating the efficacy and practicality of nested PCR, compared to standard microbiological techniques (gram stain, potassium hydroxide smear and culture) for diagnosing fungal keratitis showed that nested PCR was a sensitive method but due to the lack of sophisticated facilities in routine laboratory procedures, can serve only as complementarily and cannot replace conventional methods (15).

In our study hands were the main anatomical site of the body that were infected with fungi. Considering the chance for hands to be in contact with pollution sources and occupational factors, the increased chance of fungal infections in this anatomical site is explainable. Consistent with other studies, *Trichipyton mentagrophyes* was the predominant fungal species isolated (3,16). Also consistent with other studies, our study showed that men were affected more than females by fungal infections (17,18). However, in another study the reverse was found (3), while another study reported equal involvement of both genders (19). These differences in gender preponderance from various geographical regions could be due to different cultures, socio-economic status and climatic condition.

In the last decade the growth in the number of fungi that clinical mycologists must be able to identify has forced investigators to develop and apply new methods for fungal identification that go beyond classical phenotypic methods (19). As a consequence in the field of clinical mycology, no area is advancing faster than the application of modern molecular tools for the identification of fungi. The advancement of this area has been driven in large part by the rapid accumulation of protein and DNA sequence data, which continues unabated and accelerates with each new advance in technology and the growing need to identify a broader range of fungi. Importantly, molecular methods for fungal diagnosis and identification directly address the declining numbers of clinical mycologists because they are not dependent on classical phenotyping methods. Our results showed that nested PCR has the power to identify the increasing numbers of fungi found to produce disease in humans and animals and be used as a gold standard in the diagnosis of fungal infections, especially in immunosuppressed and immunocompromised patients. Speed of diagnosis is critical for them and should be set up in medical mycology centers as a diagnostic tool.

It would be helpful to know the percentage loss of fungal and human DNA that occurs during DNA extraction, or conversely the yield of fungal DNA detected when various concentrations of organisms are spiked in a given amount of tissue. An exogenous positive extraction control, which is phylogenetically unrelated to the target pathogen yet reasonably reflects the passage of target pathogen through the extraction route, would help monitor extraction efficiency in clinical samples. Designing the primer quality is important too (20). Meanwhile, considering different genes in different signaling pathways in different types of malignancies alongside with their epigenetics fluctuations beside the investigation of fungi is recommended.

In conclusion, the incidence of fungal infections in our study population was relatively high and dermatophytes were the most common cause of fungal infections. Nested PCR had a high sensitivity and may be useful as a reliable screening method particularly in immunosuppressed and immunocompromised patients.

ACKNOWLEDGMENTS

This study was supported by the Massoud Clinical Laboratory, Tehran, Iran which we gratefully acknowledge.

AUTHOR INFORMATION

Nasrin Sheikhi, MSc, Researcher[1]
Zahra Rafat, PhD, Assistant Professor[2]
Saeed Zaker Boostan Abad, PhD, Assistant Professor[3]
Sina Mirza Ahmadi, PhD, Assistant Professor[1]
Reza Azizian, MSc, Researcher[4]
Masoud Hamidi, PhD, Assistant Professor[5]

[1]Department of Science and Medicine, Islamic Azad University, Zanjan, Iran
[2]Tehran University of Medical Sciences, Tehran, Iran
[3]Parand Branch, Islamic Azad University, Parand, Iran
[4]Department of Medical Microbiology, Ilam University of Medical Sciences, Ilam, Iran
[5]Faculty of Paramedicine, Guilan University of Medical Sciences, Rasht, Iran.

Corresponding author: Dr. Masoud Hamidi.
E-mail: m.hamidi2008@gmail.com

REFERENCES

1. CC Kibbler, DWR Mackenzie and FC Odds (Editors). Principles and Practice of Clinical Mycology. Wiley Chichester, UK, 1996.
2. Topley WWC, Wilson GS, Collier LH, Mahy BWJ, Ter Meulen V. Topley & Wilson's Microbiology and Microbial Infections (9th Edition). Hodder Arnold, New York, 2005.

3. Rafat Z, Hashemi S, Saboor-Yaraghi AA, et al. A systematic review and meta-analysis on the epidemiology, casual agents and demographic characteristics of onychomycosis in Iran. *J Mycol Med* 2019; 29: 265-272.
4. Steinbach WJ, Mitchell TG, Schell WA, et al. Status of medical mycology education. *Med Mycol* 2003;41: 457-467.
5. Fraser VJ, Jones M, Dunkel J, et al. Candidemia in a tertiary care hospital: epidemiology, risk factors, and predictors of mortality. *Clin Infect Dis* 1992; 15: 414-421.
6. Ostrosky-Zeichner L, Pappas PG. Invasive candidiasis in the intensive care unit. *Crit Care Med* 2006; 34: 857-863.
7. Ostrosky-Zeichner L. Invasive mycoses: diagnostic challenges. *Am J Med* 2012; 125(1 Suppl): S14-S24.
8. Barnes RA. Early diagnosis of fungal infection in immunocompromised patients. *J Antimicrob Chemother* 2008; 61 Suppl 1: i3-i6.
9. Campbell MC, Stewart JL. Medical Mycology Handbook. John Wiley & Sons, 1980.
10. Haldane DJ, Robart E. A comparison of calcofluor white, potassium hydroxide, and culture for the laboratory diagnosis of superficial fungal infection. *Diagn Microbiol Infect Dis* 1990; 13: 337-339.
11. Skladny H, Buchheidt D, Baust C, et al. Specific detection of Aspergillus species in blood and bronchoalveolar lavage samples of immunocompromised patients by two-step PCR. *J Clin Microbiol* 1999; 37: 3865-3871.
12. Ajello L, Hay R, Topley WWC, Wilson SGS. Topley & Wilson's Microbiology and Microbial Infections: Medical Mycology. Hodder Arnold, 2005.
13. Andrew M, Peever TL, Pryor BM. An expanded multilocus phylogeny does not resolve morphological species within the small-spored Alternaria species complex. *Mycologia* 2009; 101: 95-109.
14. Garg J, Tilak R, Garg A, et al. Rapid detection of dermatophytes from skin and hair. *BMC Res Notes* 2009; 2: 60.
15. Badiee P, Nejabat M, Alborzi A, et al. Comparative study of Gram stain, potassium hydroxide smear, culture and nested PCR in the diagnosis of fungal keratitis. *Ophtalmic Res* 2010; 44: 251-256.
16. Surendran K, Bhat RM, Boloor R, et al. A clinical and mycological study of dermatophytic infections. *Indian J Dermatol* 2014; 59: 262-267.
17. Di Chiacchio N, Suarez MV, Madeira CL, Loureiro WR. An observational and descriptive study of the epidemiology of and therapeutic approach to onychomycosis in dermatology offices in Brazil. *An Bras Dermatol* 2013; 88 Suppl 1: 3-11.
18. Alvarez MI, González LÁ, Castro LA. Onychomycosis in Cali, Colombia. *Mycopathologia* 2004; 158: 181-186.
19. Vélez A, Linares MJ, Fenández-Roldán JC, CasalM. Study of onychomycosis in Córdoba, Spain: prevailing fungi and pattern of infection. *Mycopathologia* 1997; 137: 1-8.
20. Asadnejad Z, Alipour M, Norollahi SE. Association of the methionine synthase gene polymorphism with recurrent miscarriage in Mazandaran province, Iran. *N Z J Med Lab Sci* 2019; 73: 108-110.

BARRIE EDWARDS & ROD KENNEDY SCHOLARSHIP

The Barrie Edwards & Rod Kennedy scholarship is one of the most significant awards offered by the NZIMLS. The scholarship provides the winner with support to attend an international or national scientific meeting up to a maximum value of $7,500 for each.

Applications for this prestigious scholarship are invited from Fellows, Members and Associate Members of the NZIMLS. Applicants must be a current financial member of the NZIMLS and have been a financial member for at least two concurrent years prior to application. To be eligible applicants must make an oral presentation or present a poster as 1st author at their nominated scientific meeting.

All applications will be considered by a panel consisting of the President and Vice-President of the NZIMLS and the Editor of the New Zealand Journal of Medical Laboratory Science (who are ineligible to apply for the scholarships). The applications will be judged on your professional and academic abilities together with your participation in the profession. The panel's decision is final and no correspondence will be entered into. Application is by letter. Please address all correspondence to: **NZIMLS Executive Officer, PO Box 505, Rangiora 7440**

There is one scholarship awarded in each calendar year. Closing date is December 20th in any given year.

In your application letter please provide the following details:

- Full name, position, work address, email address and contact phone number
- The length of time you have been a financial member of the NZIMLS
- The conference you wish to attend - please provide dates
- A budget comprising airfares, conference registration and accommodation costs
- The abstract of your intended oral or poster presentation and whether it has been accepted for presentation (proof required)
- Your intentions to publish your results
- State briefly your history of participation in the profession over the last 5 years
- State the reasons why you wish to attend your nominated scientific meeting

Barrie Edwards Rod Kennedy

Successful applicants will be required to provide a full written report on return which will be published in the Journal. If not intended to publish elsewhere, successful applicants will be required to submit their study results for consideration by the New Zealand Journal of Medical Laboratory Science.

ORIGINAL ARTICLE

A New Zealand based quality assurance programme for the indirect immunofluorescence antinuclear antibody assay on HEp-2 / HEp-2000 cells. The first year.

Andrew W Soepnel

ABSTRACT

Objective: With the aim of assessing and improving harmonisation of HEp-2 based immunofluorescence antinuclear antibody testing and pattern reporting between New Zealand laboratories, a national quality assurance programme was introduced as part of the existing Waikato Quality Assurance Programmes.

Methods: Throughout 2019, five dispatches were sent to participating laboratories. Each dispatch included both a serum sample and a separate specimen-free module. Results were submitted as a final laboratory result and as individual reader results from each laboratory. Participants were encouraged to submit photos to support their serum results. Results from each dispatch were compiled into reports and returned to participants after each round.

Results: Excellent inter-laboratory agreement was seen for monospecific and simple mixed patterns. However, some significant patterns, which were supported by second-round specific antibody testing, were missed by some laboratories when present as part of a complex or mixed pattern. Importantly, supporting photos submitted by participants allowed some distinction between inherent variation attributable to (a) substrate and fixation procedures (b) subjective reading (c) conjugate type (d) lamp source and microscope type and (e) other contributory laboratory processes**.** Results from the specimen-free module showed relatively good agreement. Individual reader results indicated that intra-laboratory variation was low with 88% agreement for pattern recognition; 95% CI (77-99) and 98% agreement for titre ±1 dilution; 95% CI (96-100) between readers within the same laboratory. A hard copy specimen-free format is currently the best way to isolate and assess inter-observer variation.

Conclusions: The first year of this programme has shown inter-laboratory variation in New Zealand to be similar to that seen in overseas programmes and has demonstrated how photo submission and a hardcopy specimen free module can provide useful insights not achieved by its larger counterparts. There is a place for this small national programme to work alongside larger programmes such as RCPA and NEQAS, as laboratories strive towards improved harmonisation of results.

Key words: antinuclear antibody (ANA), HEp-2, indirect immunofluorescence, connective tissue disease, quality assurance.

N Z J Med Lab Sci 2020; 74: 119-125.

INTRODUCTION

Immunofluorescence is regarded as the gold standard methodology for antinuclear antibody (ANA) detection. This high sensitivity assay is typically the first performed for patients with suspected connective tissue disease (1). With subjective result assessment, the immunofluorescence method is challenged by both intra- and inter-laboratory variation. The International Consensus on ANA Patterns (ICAP) (www.anapatterns.org) has taken significant steps towards the harmonisation of immunofluorescence patterns (2), further work in diagnostic laboratories is required to further improve harmonisation of reported results.

In New Zealand, ten laboratories perform HEp-2 based immunofluorescence and all are enrolled in at least one international quality assurance programme (QAP). In 2019, a national ANA immunofluorescence QAP was piloted free of charge and then formally implemented by Waikato Hospital Laboratory as part of their external QAP portfolio. The dual aims of this QAP were to (a) demonstrate the level of concordance of results between New Zealand laboratories and (b) to serve as a quality improvement and training tool. A unique feature of this programme will be to collate New Zealand laboratories performance over time since there has not been a formal publication directly addressing New Zealand inter-laboratory variation. This article presents the unique features of this QAP and will provide extracts from result reports generated during the 2019 cycle.

METHODS

Factors influencing HEp-2 based immunofluorescence results can be grouped as biological, technical and operator related (3). To be a useful tool for quality improvement, a QAP should allow participants to identify the root cause for the variability of their results to one or more of those named groups. This QAP used a specimen-free module and photo submission as enablers.

Each dispatch included a serum sample to be processed routinely and a separate specimen-free module consisting of a photo set from a case at different dilutions and magnifications. Participants were encouraged to support their serum sample results with digital photos which were included in the returned reports. After the third QA cycle, participants without access to digital microscopy were invited to send their slides to the programme coordinator who performed the microscopy.

Results were submitted anonymously for individual readers within a single laboratory and as a final laboratory issued result. This provided an opportunity for every individual, who is either competent or under training in New Zealand, to submit their own interpretation of the serum and specimen-free modules each round. Laboratory names were reported alongside results to promote open discussion and accountability.

Serum samples were selected to be a realistic representation of what might be encountered routinely. A mix of weak and strong positive samples were dispatched and some very weak and negative samples will be included in future dispatches. The majority of distributed sera had multiple ANA patterns, which have been shown to challenge participants in overseas QAP modules (4,5). For future distributions, sera giving cytoplasmic staining patterns will be included.

Although target results existed for each cycle, they were not reported as they had generally only been established by one (participating) laboratory and only by their methodology. Consensus results were also not published. Due to the small number of participants in this QAP, there is a higher possibility for consensus results to be incorrect than in a larger QAP. Furthermore, if a consensus exists, it can be established simply by viewing the results table.

The pilot programme commenced in February 2019 with nine participating laboratories and consisted of two cycles. One participant used a solid phase assay instead of HEp-2 based immunofluorescence, results from this laboratory have been excluded. Participant reagents and platforms are presented in Table 1. The two remaining laboratories offering HEp-2 based ANA testing in New Zealand did not participate in the programme. A single laboratory [5] discontinued performing ANA immunofluorescence and two others [1 and 6] chose to discontinue their participation, leaving five participants [2,3,4,7 and 8] for the first round of the official programme (cycle 3). One laboratory [6] re-entered the programme for cycle 4.

RESULTS

Cycle 1

The submitted serum specimen was reactive for DFS70 antibody. Second line specific antibody testing by both line immunoassay (Euroimmun, Germany) and chemiluminescence (CMIA, Werfen Diagnostics, Spain) was positive for DFS70 IgG. By immunofluorescence methodology, the pattern was nuclear dense fine specked. Reassuringly, seven of eight (88%) laboratories either directly reported DFS70 or strongly suspected the pattern was attributable to the antigen (Table 2). A single laboratory (participant 1) scored the specimen negative. Participant 1 did not use a higher screening serum dilution as compared to other participants and belonged in the same method group as two other participants [3 and 8] who scored the serum reactive (Figure 1). For the seven laboratories reporting a titre value, the median titre was 320. All participants scored the sample within ± 1 titre of this value, which is acceptable variance.

Cycle 2

The submitted serum specimen was positive for anti-gp210, NOR90, RNA polymerase III and Ro52 IgG by line immunoassay. By immunofluorescence methodology, the pattern was mixed punctate nuclear envelope, punctate nucleolar and speckled (Figure 2). Both nuclear envelope and nucleolar staining were detectable on primate liver tissue (Euroimmun, Germany) (Figure 3).

All eight participants agreed the sample was strongly positive for an ANA with all reporting titres ≥1280 (Table 3). Five (63%) participants [3,4,5,6 and 8] reported a nuclear envelope pattern either directly or by other acceptable nomenclature. One (13%) laboratory [3] reported the nucleolar pattern. Nucleolar organiser region staining was either not reported or misinterpreted as nuclear dots by all other laboratories. Two participants [2 and 7] were in agreement, but incorrect. All other participants returned different combinations of mixed patterns and only one [3] correctly identified the three ANA patterns supported by line immunoassay results (nuclear envelope, nucleolar and speckled).

Cycle 3

The submitted serum was negative by CMIA CTD screen (Werfen Diagnostics, Spain), radioimmunoassay for anti-dsDNA (IBL, Germany) and by extractable nuclear antigen, inflammatory myopathy and extended liver line immunoassay panels (Euroimmun, Germany). By immunofluorescence methodology, the pattern was speckled, with a mix of nuclear fine speckling and small dots in each cell (Figure 4a). The dots were not typical of a multiple nuclear dots or centromere pattern.

Four of five laboratories (80%) reported a speckled or atypical speckled pattern only. The remaining laboratory (participant 2) reported multiple nuclear dots as the primary pattern and speckled as a weaker second pattern. The median titre for the speckled pattern was 160 and all participants scored the sample within ± 1 titre of this value.

Cycle 3 specimen-free module

The submitted photo set was of a homogeneous and speckled pattern by immunofluorescence on HEp-20-10 cells (Euroimmun, Germany), represented in six images at dilutions ranging from 160 to 2560 and at 200x, 400x and 630x magnification. Colour prints were provided in A4 size hardcopy only and a negative anti-DFS70 IgG test result was provided.

Four of five laboratories (80%) reported a homogeneous and speckled pattern. The remaining laboratory (participant 2) noted the homogenous and speckled pattern but reported it as homogeneous as per their laboratory protocol when anti-DFS70 specific testing is negative. The median titre was 640. A single laboratory (participant 8) scored the titre at -2 dilutions from this value, while all other results were within ±1 titre.

Cycle 4

The submitted serum was positive for anti-centromere protein A and B, Ro-52, and SSA by line immunoassay, and anti-dsDNA by radioimmunoassay. By immunofluorescence methodology the pattern was homogeneous and centromere. The centromere staining was partially masked by strong homogeneous staining at the screening dilution but was identifiable at higher dilutions (Figure 5).

Five of six of laboratories (83%) reported both homogeneous and centromere patterns. A single laboratory (participant 7) reported a homogeneous pattern only. In addition to the four individual readers from laboratory 7, a further four readers from two other laboratories [2 and 3] did not report centromere pattern (40% of readers overall). The median titre for the homogeneous and centromere mixed pattern was 1280 and all participants scored the sample within ± 1 titre of this value.

Individual reader results

Intra-laboratory variation is summarised in Table 4. Overall, intra-laboratory variation was very low with agreement for pattern and titre results ranging between 70% (cycle 3) to 96% (cycle 1). The mean agreement between readers within the same laboratory was 88% for ANA pattern, 95% CI (77-99) and 98% for titre ±1 dilution, 95% CI (96-100).

DISCUSSION

Overall variation and proportion of incorrect results in New Zealand was comparable with that of larger overseas programmes when complexity of the ANA pattern is considered. The proportion of acceptable results in cycles 1 and 3 were 88% and 80% respectively, similar to overseas QAP reports where simple and monospecific patterns have been examined. Up to 78% of laboratories in the South Korean national QAP achieved correct results with a monospecific dense fine speckled pattern (4). In the large Belgian national QAP, Van Blerk *et al.* defined acceptable limits as median titre +/- two doubling dilutions and a wide range of titre results were reported, usually with about 90% within these limits (5).

Another simple pattern was submitted for the cycle 3 specimen-free module and again 80% of responses were acceptable with regards to reported pattern. This is as expected based on the highest agreement in pattern recognition reported by Rigon *et al.* between six experts reading a large set of digital images, which was 84% for positive and 43% for weakly positive samples (6).

Poorer conformity of results was expected for the more complex ANA patterns submitted for cycles 2 and 4. A monospecific centromere pattern was recognised at a rate of 83% in the Belgian QAP in 1992, then 97.6% in 2003 (5). In cycle 4, one of six laboratories and 40% of readers missed the centromere pattern. It is important, however, to recognise this was present as part of a mixed pattern. Jearn and Kim reported much lower rates of correct pattern recognition in polyspecific samples, as low as 73.5% for centromere, compared to 96.9%

for monospecific centromere (4). Cycle 2 was the most complex ANA pattern submitted in the current QAP and the inter-laboratory variation and proportion of acceptable results was much poorer than all other cycles, but still consistent with reports from other QAPs for mixed patterns (5,6).

Analysis of submitted micrographs from cycles 2 and 4 suggests that subjective reading was the main source of inter-laboratory variation. Key features of the ANA pattern submitted in cycle 2 were a negative chromatin plate in metaphase cells, coarse grainy staining of interphase cells with a pitted, slightly more intensely stained outer rim and accentuation of fluorescence where nuclei overlap (Figure 2). These are the hallmarks of a punctate nuclear envelope pattern (2). A punctate nucleolar pattern is also identifiable by characteristic nucleolar organiser region staining in metaphase chromosomal plates (2) and in diluted serum, interphase cell nucleolar staining is visible, albeit weak and somewhat obscured by the nuclear envelope staining (Figure 2).

Anti-RNA polymerase III and Ro52 IgG were detected by line immunoassay, which supports readers who identified a speckled pattern. This also provides further support for the punctate nucleolar pattern because anti-RNA polymerase I and III often co-exist (7), anti-RNA polymerase III can be associated with a coarse speckled pattern, while anti-RNA polymerase I is associated with a punctate nucleolar pattern (2).

Evidence of nucleolar organiser region, nucleolar and nuclear membrane staining was visible in all submitted micrographs (participants 2, 3 and 8), indicating that pattern recognition by slide readers was a significant source of error. Increased participation in micrograph submission by HEp-2000 slide users will be required to confirm this in future cycles.

Pattern recognition by slide readers was also the most likely source of error in cycle 4. No photos were submitted by the laboratory that did not report a centromere pattern (participant 7). However, centromere staining was clearly visible in all photos submitted, including those from a laboratory using the same slides and put up platform (participant 6).

While reader subjectivity also appears to have contributed to the false-negative result submitted by participant 1 in cycle 1, submitted micrographs reveal methodology to be a greater contributing factor in this case. Figure 1 shows the remarkable difference in fluorescence intensity observed by participants 1 and 8. Despite sharing the same HEp-2 slide manufacturer and pre-analysis robotics, difference in method performance contributed significantly to the false negative result reported by participant 1. With this knowledge participant 1 should be able to perform a more targeted investigation into this error.

Methodology, specifically substrate used, was also shown to influence the reported ANA pattern in cycle 3. Micrographs submitted by participants 3 and 8 were of a speckled ANA pattern, where some cells showed discrete dots that were not bold or consistent enough across the slide to constitute a multiple nuclear dots pattern. However, micrographs submitted by participant 2, the one laboratory that reported nuclear dots as the primary pattern and the only Bio-Rad Kallestad slide user, showed an ANA pattern with far more prominent dots, which could understandably be interpreted as either speckled or multiple nuclear dots (Figure 4b).

A lack of standardisation between HEp-2 based immunofluorescence assays has been established previously. Copple *et al*. reported that the overall agreement between slides from five manufacturers tested with the same serum samples and read by the same panel of readers, ranged between 44% for a scleroderma patient group and 93% for healthy donor samples (8). Conversely, in a much larger analysis of QAP results by Van Blerk *et al*., there was no association between pattern results and slide manufacturers (5). However, as the authors acknowledged, this was probably because only basic, monospecific patterns were analysed. Further analysis of a large number of samples tested by all substrates in use is required to determine if substrate has a significant influence on ANA pattern results in New Zealand. Over time this will be achieved by the current QAP.

The submission of unacceptable results in this QAP for samples where the ANA result could significantly influence clinical decisions has highlighted inherent risk with immunofluorescence methodology. For example, the dense fine speckled pattern was missed by participant 1 in cycle 1. The detection of a dense fine speckled pattern is important because it prompts follow up testing by a DFS70-specific assay. Anti-DFS70 IgG, when detected as an isolated autoantibody, is thought to be an exclusion marker for connective tissue disease (9).

Similarly, the centromere pattern was missed by participant 7 in cycle 4. Centromere antibodies are associated with the limited form of systemic sclerosis and the detection of this pattern has diagnostic and prognostic value. Identification of a centromere pattern is particularly important because it is included in the classification criteria for systemic sclerosis, one of the few situations where an ANA pattern is in itself an important factor in the diagnosis (10).

A key objective of this QAP is to serve as a quality improvement and training tool. The examples show there is both room for improvement in New Zealand and that such improvement would have clinical relevance.

Intra-laboratory variation

Our programme lacked sufficient participant numbers to produce statistically relevant data, but the recording of individual reader results at each laboratory provides an impression of the level of inter-reader subjectivity within New Zealand laboratories. Intra-laboratory agreement for ANA patterns ranged between 70-96%. This is higher than the highest inter-reader agreement observed by Rigon *et al*. between experts reading the same digital images, which were 77.9% for homogeneous patterns and 22% for a combined pattern (6). Confounding variables associated with reading from digital images, such as monitor settings and digital zoom, may have contributed to the variation seen in their study as control of these factors was not mentioned.

Possible reasons for the high intra-laboratory agreement in New Zealand are a single senior trainer within each laboratory leading to conformity of less experienced readers and the possibility of a dominant reader influencing less confident reader results if double-blind reading is not strictly practised. These are 'real world' factors not present in double-blind studies involving only expert readers.

Limitations and future prospects

The success of this programme depends on the number of laboratories participating and the extent to which they participate with regards to photo and individual reader result submission.

Photo submission is the primary feature of this programme that sets it apart from the QAPs already in use in New Zealand. The average rate of photo submission was 50%. In future, participating laboratories will be more actively encouraged to submit photos or slides.

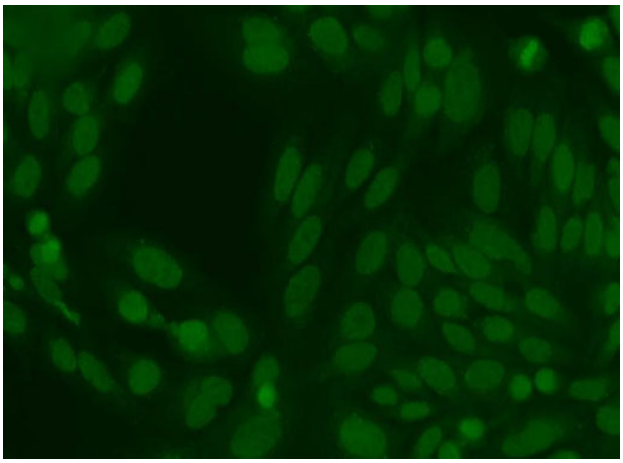
Reporting was limited to compilation and presentation of results in a concise format. Links to related articles and commentary from the programme coordinator were occasionally included. Future reports may expand this element to provide a better educational resource and to promote discussion between participants at scientific meetings. Such advice from QAP providers has been associated with reduced inter-laboratory variation over time (5,11).

Increased use of automated readers and computer-aided diagnosis systems is expected to reduce reader subjectivity and intra-laboratory variation (3). QAPs should continue to assess inter-laboratory variability but only those that feature micrograph submission will be able to distinguish between variability caused by methodology and subjective interpretation, either by computer-aided diagnosis systems or users. Ideally, future programmes should allow participants to upload raw image files from their automated slide readers to be analysed at each location, thus truly isolating the sources of variation.

CONCLUSIONS

The Waikato QAP has provided an indication of inter-laboratory variability of ANA detection by immunofluorescence in New Zealand. This programme is unique in that it used micrograph submission and a hardcopy specimen-free module to narrow down the sources of variation, a level of detail not routinely provided by other QAPs. Individual result submission has allowed for a good first impression of intra-laboratory variability in New Zealand laboratories. It is hoped that this programme, alongside ICAP and the existing QAPs, will play an important role in improving harmonisation of ANA testing by immunofluorescence in New Zealand.

Laboratory 1 at 1:80 dilution.



Laboratory 8 at 1:100 dilution.

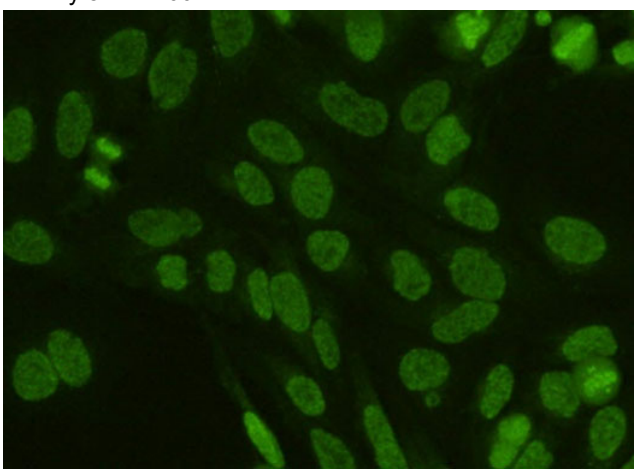
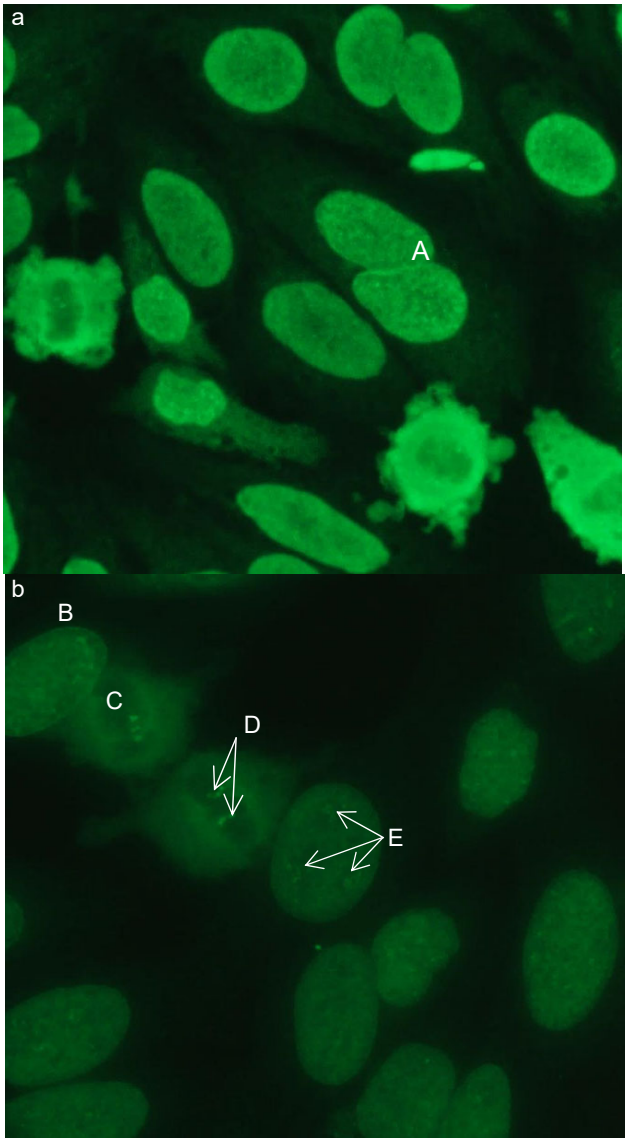


Figure 1. Submitted micrographs from cycle 1. Both laboratories used Euroimmun HEp-20-10 cells with slide preparation by Euroimmun Sprinter platforms.



Figures 2a (1:640) and 2b (1:2560). Euroimmun HEp-20-10 cells showing the ANA pattern in cycle 2 with features of punctate nucle-ar envelope (A,B,C) and punctate nucleolar (D,E) patterns.
A - Accentuation of fluorescence where nuclei overlap. B - Pitted, slightly more intensely stained outer rim. C - Negative chromatin plate. D - NOR staining in metaphase cells. E - Punctate nucleolar staining.

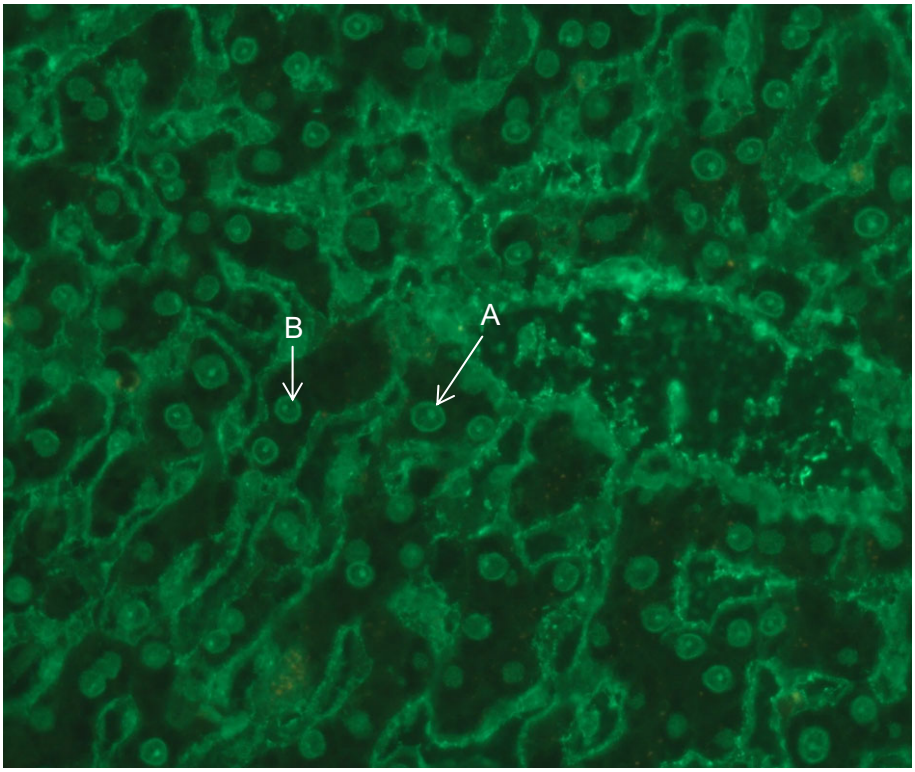
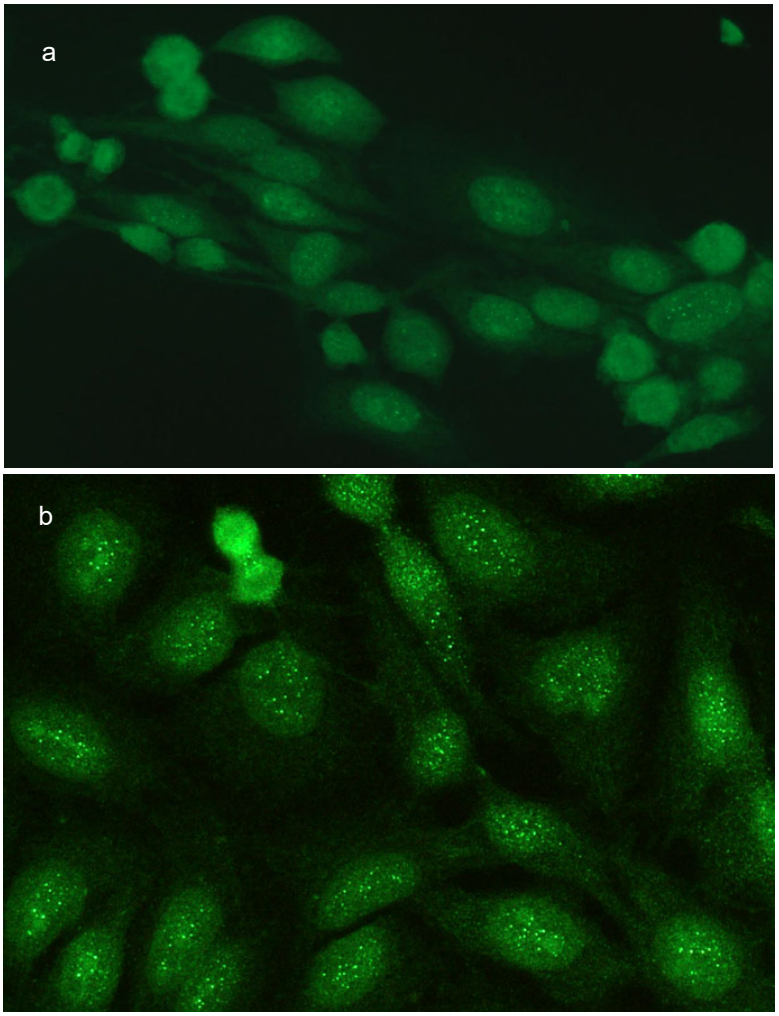


Figure 3. Euroimmun primate liver tissue at 1:640 dilution with serum from cycle 2. This supplementary substrate included on Euroimmun mosaic slides clearly demonstrates nucleolar and nuclear membrane staining.
A - Nucleolar staining. B - More intensely stained outer nuclear rim.



Figures 4a and 4b. The dots in the speckled ANA pattern from cycle 3 are more prominent when tested with Bio-Rad Kellestad slides than on Euroimmun slides.
a – Euroimmun HEp-20-10 cells at a 1:80 dilution (laboratory 3). b – Bio-Rad Kallestad cells at a 1:80 dilution (laboratory 2).

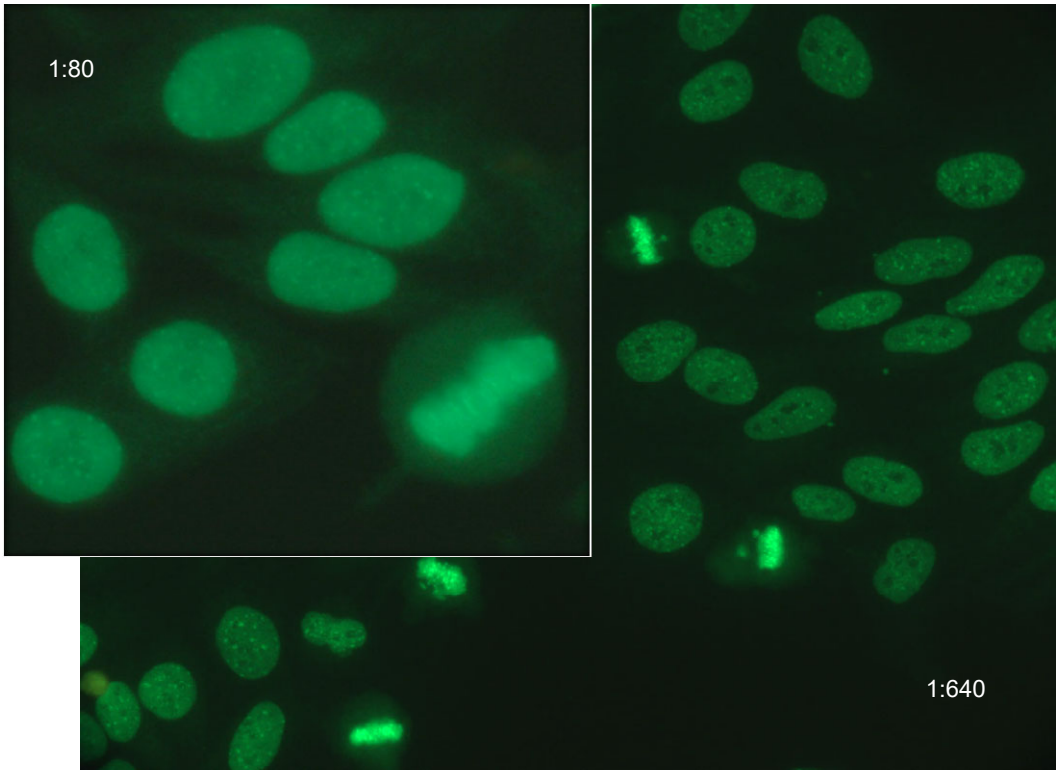


Figure 5. Submitted micrographs from laboratory 3 for cycle 4. Euroimmun HEp-20-10 cells show partially masked centromere staining at a 1:80 dilution which shows through clearly at 1:640.

Table 1. Participants method and reagent details.

Lab	Screening dilution(s)	Slides	Slide put up	Slide read
1	80	Euroimmun HEp-20-10	Sprinter	Europattern
2	80	Bio-Rad Kallestad HEp-2	DAS AP22 Speedy IF	Manual
3	80	Euroimmun Mosaic: HEp-20-10 / Liver (Monkey)	Manual	Manual
4	80 & 320	HEp-2000	DSR	Manual
5	80	HEp-2000	Manual	Manual
6	80 & 320	HEp-2000	DSRie	Manual
7	80 & 320	HEp-2000	DSR	Manual
8	100	Euroimmun Mosaic: HEp-20-10 / Liver (Monkey)	Sprinter	Manual

Table 2. Cycle 1 results.

Lab	ANA Screen	Pattern	Titre	Notes
1	Negative			
2	Positive	DFS70	160	DFS70 confirmed by immunoblot.
3	Positive	DFS70	160	DFS70 confirmed by Bioflash. Would also reflex ENA and dsDNA. Reported comment depends on all 3 results.
4	Positive	Homogeneous/Speckled ?DFS70	320	
5	Positive	?DFS	320	All suspected DFS samples referred to reference laboratory for confirmation of pattern and titre.
6	Positive	DFS70	320	DFS confirmed on Bioflash. Would also reflex ENA and DNA.
7	Positive	Homogeneous/Speckled ?DFS70	640	Suspect DFS70. Would get confirmation plus ENA & dsDNA. Would report as DFS70 if confirmed, or HSp if negative.
8	Positive	DFS70	640	DFS70 confirmed by blot.

DFS=dense fine speckled.

Table 3. Cycle 2 results.

Lab	ANA Screen	Pattern 1	Titre 1	Pattern 2	Titre 2	Pattern 3	Titre 3
2	Positive	Speckled	>1280				
7	Positive	Speckled	>2560				
1	Positive	Speckled	1280	Homogeneous	1280		
8	Positive	Speckled	>1280	Nuclear membrane	>1280		
6	Positive	Nuclear pore	>1280				
5	Positive	Nuclear envelope	>1280	Multiple nuclear dots	>1280		
4	Positive	Speckled	5120	Nuclear membrane	5120	Mitotic dots	5120
3	Positive	Speckled	2560	Nuclear membrane	>2560	Nucleolar	>2560

Table 4. Intra-laboratory variation of immunofluorescence ANA reading during the first year of the Waikato QAP.

QAP cycle	# of individual readers	# of readers out of own laboratory consensus (pattern)	# of readers out of own laboratory consensus (titre >±1 dilution)
1	26	1 (4%)	1 (4%)
2	23	4 (17%)	0
3	20	6 (30%)	1 (5%)
3 specimen-free	19	1 (5%)	0
4	20	4 (20%)	0

ACKNOWLEDGEMENTS

Waikato QAP acknowledges the important work of the participating laboratories and commends their commitment to service quality: Canterbury SCL, Dunedin Hospital SCL, Labtests, Medlab Central, Middlemore Hospital, Pathlab Waikato, Taranaki Medlab, Waikato Hospital, and Wellington Hospital SCL. Thank you to my colleagues, Dr. Marianne Empson, Jenny Bennett, Sarah Hall and Dr. Stephen du Toit for kindly reviewing this manuscript.

AUTHOR INFORMATION

Andrew W Soepnel, BMLSc MSc, Technical Specialist
Immunology Department, Waikato Hospital, Hamilton

Correspondence: Andrew Soepnel.
Email: andrew.soepnel@waikatodhb.health.nz

REFERENCES

1. Meroni PL, Schur PH. ANA screening: an old test with new recommendations. *Ann Rheum Dis* 2010; 69: 1420-1422.
2. Chan EKL, Damoiseaux J, Carballo OG, et al. Report of the First International Consensus on Standardized Nomenclature of Antinuclear Antibody HEp-2 Cell Patterns 2014–2015. *Front Immunol* 2015; 6: 412.
3. Infantino M, Meacci F, Grossi V, et al. The burden of the variability introduced by the HEp-2 assay kit and the CAD system in ANA indirect immunofluorescence test. *Immunol Res* 2017; 65: 345-354.
4. Jearn LH, Kim TY. Should the external quality assessment sample of antinuclear antibodies be of a typical monospecific pattern? *Clin Chem Lab Med* 2010; 48: 1189 -1190.
5. Van Blerk M, Van Campenhout C, Bossuyt X, et al. Current practices in antinuclear antibody testing: results from the Belgian External Quality Assessment Scheme. *Clin Chem Lab Med* 2009; 47: 102-108.
6. Rigon A, Infantino M, Merone M, et al. The inter-observer reading variability in anti-nuclear antibodies indirect (ANA) immunofluorescence test: A multicenter evaluation and a review of the literature. *Autoimmun Rev* 2017; 16: 1224-1229.
7. Mehra S, Walker J, Patterson K, Fritzler MJ. Autoantibodies in systemic sclerosis. *Autoimmun Rev* 2013; 12: 340-354.
8. Copple SS, Giles SR, Jaskowski TD, et al. Screening for IgG antinuclear autoantibodies by HEp-2 indirect fluorescent antibody assays and the need for standardization. *Am J Clin Pathol* 2012; 137: 825-830.
9. Ochs RL, Mahler M, Basu A, et al. The significance of autoantibodies to DFS70/LEDGFp75 in health and disease: integrating basic science with clinical understanding. *Clin Exp Med* 2016; 16: 273-293.
10. van den Hoogen F, Khanna D, Fransen J, et al. 2013 Classification criteria for systemic sclerosis: an American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* 2013; 65: 2737-2747.
11. Pham BN, Albarede S, Guyard A, et al. Impact of external quality assessment on antinuclear antibody detection performance. *Lupus* 2005; 14: 113-119.

BRIEF COMMUNICATION

Gender trends in authorship of articles in the New Zealand Journal of Medical Laboratory Science: 1995-2019

Rob Siebers

ABSTRACT

Objectives: Gender imbalances have been shown in articles in medical journals. No such information exists for medical laboratory science journals.
Methods: Retrospective analysis of all peer-reviewed articles of the New Zealand Journal of Medical Laboratory Science from 1995 to 2019. Gender of all named authors was recorded as well as for first or senior authors.
Results: A total of 284 articles were published with 650 listed authors, 267 (41.1%) who were female. Of these 267 female authors, 156 were listed as first or senior author (49.1% of first/senior authors). Overall, female authorship increased from 1995 to 2014 (39.0% to 46.3%) but then declined in the 2015-2019 period (36.5%). However, New Zealand female authorship has increased since 2005 to around 55% while New Zealand females comprised more than 60% of first or senior authors since 2010.
Conclusions: Overall, female authors have been under-represented in publications in the New Zealand Journal of Medical Laboratory Science in the past, but this has increased, especially New Zealand females as first or senior authors.
Keywords: publication, authorship, gender, medical laboratory science, New Zealand.

N Z J Med Lab Sci 2020; 74: 126-128.

INTRODUCTION

Publications in journals are important for academics, not only for reporting research but also for promotion and grant funding. Several studies have shown that females are under-represented as authors in the medical sciences. These studies have generally been in specialist medical journals (1-5). However, no such studies have been published in medical laboratory science journals. Staff of medical laboratories are predominantly female, especially in New Zealand and most laboratory staff are not academics.

Therefore, a study was conducted to determine whether there were any gender imbalances in publications from the New Zealand Journal of Medical Laboratory Science, a peer-reviewed journal publishing on all topics of medical laboratory science.

METHODS

In this retrospective study all peer-reviewed articles published in the New Zealand Journal of Medical Laboratory Science between 1995 and 2019 were retrieved from the journal's website (6). Included were all peer reviewed articles, including review and viewpoint articles. Excluded were editorials, letters, book reviews and reports. The gender of each listed author as well as the gender of the first or senior author was recorded. Additionally, it was recorded if any author's affiliation was from New Zealand. Gender was determined from the authors first or given names. Where gender was not apparent from the authors first or given names, a Google search was performed using a name-gender association and country of origin of the author. Recorded were the number of total articles and those with at least one New Zealand based author.

RESULTS

In the 25-year period (1995-2019) 338 articles were published of which 262 articles (77.5%) had at least one New Zealand author and 76 articles were exclusively from overseas authors. Articles from overseas increased substantially from 21 articles during 1995 to 2009, to 55 articles from 2010 to 2019.

Over the 25-year period 40.7% of all named authors were female and 47.9% of females were listed as first or senior author. Female authorship increased from 38.8% in the 1995-1999 period to 45.8% in the 2010-2014 period before declining in the 2015-2019 period (Table 1). Female first or senior author status varied over 25 years, ranging between 44.6% (2010-2014) and 54.0% (2005-2009). When analysis was restricted to New Zealand only authors, a higher proportion of females were named authors (47.8%) and first or senior author (56.5%) (Table 2).

DISCUSSION

Over the years various studies have documented gender imbalances in published articles, predominantly in the medical literature. Most have shown a gender imbalance in that women were underrepresented as first or senior authors in peer reviewed journals. Jagsi *et al.* showed that women composed a minority of first authors in six major USA medical journals, although that increased from 5.9% in 1970 to 29.3% in 29.3% in 2004 (1). A recent study showed that, although first female authorship increased in high impact medical journals over time, this has plateaued and even declined in some journals (2). However, in another study it was shown that women published research as first or senior author at a rate proportional to the number of academic female physicians (3). In other medical specialities gender disparities in female authorship have been demonstrated. For instance, in paediatrics females were underrepresented as first author, although an increase was noted over time (4). Likewise, in academic obstetrics and gynaecology in the USA, where women outnumber men, female first and senior authorship were underrepresented although this increased significantly over the years, especially female first authorship after 2000 (5).

The gender gap in authorship is not confined to the medical literature alone. For instance, Giuffrida *et al.* showed that gender disparities in authorship exists in the veterinary literature (7). To our knowledge, no studies on gender and authorship have been examined in the medical laboratory science literature. Our study has shown that females are underrepresented as authors in the New Zealand Journal of Medical Laboratory Science, not only as first or senior author, but also as a named author. This is despite that in 2015 76% of medical laboratory scientists and 86% of medical laboratory technicians of New Zealand District Health Boards laboratories in the workforce were female (9). Female representation in medical laboratories overseas is also high at >70% (9-11).

Overall, female authorship increased in the New Zealand Journal of Medical Laboratory Science from 1995 to 2014, but they declined in the 2015-2019 period. This was most likely due to an increase in overseas articles submitted since the journal became open access in 2011 (12) as overseas authors of published articles were predominantly male (unpublished observations). However, overall New Zealand female authorship slightly declined in this period, as did first or senior female authorship.

The present study did not determine reasons for the gender disparity in authorship. One reason could be editorial gender bias, however, during the period of study no New Zealand submitted articles were rejected for publication and a recent study has also found that editorial gender bias was unlikely to contribute to gender differences in authorship in evolutionary journals (13). Another possible reason could be an unconscious gender bias that exists in the scientific community (14). It is also possible that females have been disproportionally excluded from authorship as authorship issues have been reported in academia (15).

In conclusion, females have been underrepresented as authors, including as first or senior author, in published articles in the New Zealand Journals of Medical Laboratory Science in the last 25 years.

Table 1. Overall authorship numbers by gender.

Time period	Male author	Female author	1st or senior male author	1st or senior female author
1995-1999	85 (61.2%)	54 (38.8%)	47 (54.0%)	40 (46.0%)
2000-2004	47 (60.3%)	31 (39.7%)	26 (50.0%)	26 (50.0%)
2005-2009	58 (58.0%)	42 (42.0%)	23 (46.0%)	27 (54.0%)
2010-2014	97 (54.2%)	82 (45.8%)	41 (55.4%)	33 (44.6%)
2015-2019	110 (63.2%)	64 (36.8%)	39 52.0%)	36 (48.0%)
1995-2019	397 (59.3%)	273 (40.7%)	176 (52.1%)	162 (47.9%)

Table 2. New Zealand authorship numbers by gender.

Time period	Male author	Female author	1st or senior male author	1st or senior female author
1995-1999	80 (61.5%)	50 (38.5%)	43 (53.7%)	37 (46.3%)
2000-2004	43 (59.7%)	29 (40.3%)	22 (47.8%)	24 (52.2%)
2005-2009	20 (45.5%)	24 (54.5%)	17 (40.5%)	25 (59.5%)
2010-2014	54 (45.4%)	65 (54.6%)	15 (31.9%)	32 (68.1%)
2015-2019	38 (44.7%)	47 (55.3%)	17 (36.2%)	30 (63.8%)
1995-2019	235 (52.2%)	215 (47.8%)	114 (43.5%)	148 (56.5%)

AUTHOR INFORMATION

Rob Siebers, PGCertPH FNZIC FNZIMLS FRSB, Associate Professor[1] and Editor[2]

[1]Wellington School of Medicine and Health Sciences, University of Otago, Wellington
[2]New Zealand Institute of Medical Laboratory Science, Rangiora

Correspondence: Rob Siebers. Email: rob.siebers@otago.ac.nz

CONFLICT OF INTEREST

Rob Siebers is the Editor of the New Zealand Journal of Medical Laboratory Science. He had no input in reviewer selection or editorial decision. This was handled independently by the journal's Deputy Editor, Michael Legge.

REFERENCES

1. Jagsi R, Guancial EA, Worobey CC, Henault LE, Chang Y, Starr R, et al. The "gender gap" in authorship of academic medical literature-a 35-year perspective. *N Engl J Med* 2006; 355: 281-287.
2. Filardo G, da Graca B, Sass DM, Pollock BD, Smith EB, Martinez MA. Trends and comparison of female first authorship in high impact medical journals: observational study (1994-2014). *BMJ* 2016; 352: i847.
3. Bernadi K, Lyons NB, Huang L, Holihan JL, Olavarria OA, Martin AC, et al. Gender disparity in authorship of peer-reviewed medical publications. *Am J Med Sci* 2019; pii: S0002-9629(19)30398-2
4. Fishman M, Williams WA 2nd, Goodman DM, Ross LF. Gender differences in the authorship of original research in pediatric journals, 2001-2016. *J Pediatr* 2017; 191: 244-249.

5. Penn CA, Ebbott JA, Larach DB, Hesson AM, Waljee JF, Larach MG. The gender authorship gap in gynecologic oncology research. *Gynecol Oncol Rep* 2019; 29: 83-84.
6. Journal Archive. New Zealand Institute of Medical Laboratory Science. https://www.nzimls.org.nz/journal-archive.html,
7. Giuffrida MA, Burton JH, Dechant JE, Winter A. Gender imbalance in authorship of veterinary literature: 1995 versus 2015. *J Vet Med Educ* 2019; 46: 429-437.
8. Workforce information. Medical laboratory workers-April 2016. Strategic Workforce Services. DHBSS-April 2016.
9. Administration, National Center for Health Workforce Analysis, 2017. Sex, race, and ethnic diversity of U.S. health occupations (2011-2015). U.S. Department of Health and Human Services, Rockville, Maryland.
10. Victorian Allied Health Workforce Research Program. Medical laboratory science workforce in Victoria, Australia. Medical Laboratory Science Workforce Report, March 2018. Department of Health and Human Services, State of Victoria.
11. Grant MM. What's sex got to do with it? Gender-based research and the Canadian medical laboratory profession. *Can J Med Lab Sci* 2003; 65: 137-140.
12. Siebers R. Open access of the Journal. *N Z J Med Lab Sci* 2011; 65(1): 4.
13. Edwards HA, Schroeder J, Dugdale HL, Gender differences in authorship are not associated with publication bias in an evolutionary journal. *PLoS One* 2018; 13: e0201725 [erratum in *PLoS One* 2019; 14: e021725].
14. Ceci SJ, Williams WM, Barnett SM. Women's underrepresentation in science: sociocultural and biological considerations. *Psychol Bull* 2009; 135: 218-261.
15. Mitcheson H, Collings S, Siebers R. Authorship issues at a New Zealand academic institution. *Int J Occup Environ Med* 2011; 2: 164-169.

Expressions of Interest for Editor of the New Zealand Journal of Medical Laboratory Science

The NZIMLS Council hereby calls for expressions of interest from NZIMLS members to succeed Rob Siebers as Editor of the New Zealand Journal of Medical Laboratory Science. Rob has been Editor since 1994 and it is timely to begin succession planning for this position. The successful person would join the Editorial Board as Deputy Editor and over a period of time would learn, under the guidance of Rob, all aspects of editorial work associated with the Journal. The Deputy Editor would take over as Editor when fully conversant with this role. The Editor will be a contractor to the NZIMLS for which remuneration will be offered. The time commitment is approximately six hours per week.

The Journal has been continuously in existence since 1948 and currently is published three times a year (April, August, and November). All submissions are peer reviewed and last year 13 articles plus a number of editorials were published.

To register your expression of interest, please write to the NZIMLS Executive Officer at fran@nzimls.org.nz. Include your current position and work place, why you are interested in this role, your experience in publishing scientific articles. Please also submit a CV.

Further information on this position may be obtained from Rob Siebers at rob.siebers@otago.ac.nz.

ORIGINAL ARTICLE

Differences in micronutrient levels between urban and rural children in Cross River State, Nigeria

Nkechi Udo, Iya E Bassey, Uwem O Akpan, Idongesit KP Isong, Faith A Effa and Augusta Ndudi

ABSTRACT

Objectives: This study assessed levels of serum vitamin A, iodine, zinc, iron, selenium, total protein, albumin and globulins in children in urban and rural areas in Cross River State, Nigeria.

Methods: A school-based, cross-sectional study was conducted on 250 school-aged children of both sexes aged 7-12 years. Two groups of 125 children were each recruited from an urban area (Calabar) and a rural area (Akpabuyo). Serum vitamin A, iodine, selenium and zinc levels were assessed by high performance liquid chromatography and total protein, albumin and iron by colorimetric methods. Anthropometric indices were evaluated and BMI-for-age determined using WHO anthroplus software. Data was analysed by Students't-test and chi-square analysis and statistical significance set at $p<0.05$.

Results: Urban children had significantly higher BMI, total protein, albumin and selenium levels, while they had significantly lower globulin, vitamin A, zinc and iodine levels, compared to rural children. The rural children had a significantly higher frequency of malnutrition (46.4%) compared to the urban children (29.6%). Urban children also had significantly higher deficiencies of iron and zinc. There was no vitamin A deficiency in either group.

Conclusions: Urban children had higher levels of macronutrients and lower levels of micronutrients than rural children. To effectively tackle nutritional deficiencies in school-aged children, nutritional programs should be oriented to suit local needs.

Key words: children, nutritional status, micronutrients, malnutrition.

N Z J Med Lab Sci 2020; 74: 129-134.

INTRODUCTION

The implementation of nutritional health programs in most countries are geared towards improving the health status of mothers and especially children, who are regarded as vulnerable groups (1). However, a severe limitation in the implementation of nutritional health programs for children is the "blanket" format applied; not taking into consideration specific needs of children in different regions, for instance, urban and rural children. The assessment of nutritional status of children from different regions can provide this information (2). It is known that lifestyle, environment, cultural and socioeconomic factors can influence the quantity and quality of food and access to healthcare available to the children from these different groups. These may impact their nutritional and micronutrient status differently (3). The Nigerian Population Commission and ICF International reported prevalence rates in Cross River State for wasting, stunting and underweight as 9.8%, 21.7% and 14.8% respectively (4).

Micronutrients are nutrients required in minute specific quantities. Most of these are not generated in the body but are derived from food intake (5). Micronutrient deficiencies are particularly relevant in children because they in a phase where there is rapid growth and development and they need these micronutrients for proper development (6).

Several studies on nutritional and micronutrient status of children in Nigeria have been carried out (7-10), including in Cross River State (11-13). However, it is some years since these studies were carried out in Cross River State and most studies on nutritional status and micronutrient status available for this region were for children aged 0 – 5 years. Therefore, this study assessed serum vitamin A, iodine, zinc, iron, selenium, total protein, albumin and globulin levels in school children aged 7 to 12years in urban and rural areas in Cross River State.

METHODS

Study design and area

This was a school-based cross-sectional study that was conducted in Calabar metropolis and Ikot Nakanda and Ikot Ene villages both located in Cross River State. The study was carried out on 250 school children aged 7-12 years of both sexes. 125 subjects schooling in Calabar metropolis and 125 from rural communities (Ikot Nakanda and Ikot Ene villages) in Akpabuyo Local Government Area in Cross River State. The children were consecutively recruited from six primary schools (three each from the urban and rural areas). The schools were chosen randomly from a list of schools in both areas obtained from the Cross River State Ministry of Health. Ethical approval was obtained from the Cross River State Ministry of Health. Written consent from parents/ guardians and oral consent from children were obtained before enrollment in the study. Data on age, sex, food consumption patterns were obtained from the subjects through a structured questionnaire. Children whose ages were below seven years and above twelve years, were chronically ill, or were girls who had started menstruating were excluded.

Sample size determination

The sample size was calculated by the Kish formula (14) using the variable in our study with the highest prevalence (stunting) of 21.7% (7), which returned a sample size of 250.

Sample collection

Five ml of blood was collected from each subject into plain sample bottles, allowed to clot and centrifuged at 3000rpm for 5minutes. Sera were separated into aliquots and stored frozen at -20^{0}C until analysis.

Anthropometric measurements

Weight to the nearest 0.1 kg (minimum clothing and without shoes) and height to the nearest 0.1 cm of each subject were measured. Body mass index (BMI) was computed as a ratio of weight (kilograms) and height (metres^2) . Data on height, weight, BMI, age and gender were used to calculate standardised nutritional indices (Z-scores) using WHO anthroPlus software v1.0.4 (WHO, Geneva, Switzerland). A software was used for the global application of the WHO reference 2007 for children aged 5 – 19 years to monitor the growth of school age children and adolescents. Overweight (> + 1SD BMI-for-age Z score), obesity (> + 2SD BMI-for-age Z score), thinness/wasting (< −2SD of BMI-for-age Z score), Normal (≤+1SD and ≥-2SD BMI-for-age Z score), underweight (< −2SD of weight-for-age Z score) and stunting (< −2SD of height-for-age (HAZ) Z score) were defined according to WHO criteria (15).

Criteria used to define protein and micronutrient deficiencies

Cut-off values for total protein and albumin deficiencies were defined as values less than 62g/L and 36g/L, respectively (16). Cut-off values for serum zinc were defined as values less than 9.9 µmol/L for children ≤10 years and 10.7 µmol/L for children >10 years (17). Cut-off values for serum iron, selenium, iodine and vitamin A were less than 10.7 µmol/L, 0.9 µmol/L, 0.33 nmol/L and 0.7 µmol/L, respectively (16,18-21).

Determination of serum micronutrients

Vitamin A levels were estimated by high performance liquid chromatography (Waters 616/626 HPLC, Waters Ltd, United Kingdom) at the International Institute of Tropical Agriculture, Ibadan, Nigeria. Serum iodine was estimated using the modified method of Sandell-Kolthoff as described by Pino *et al.* (22). Concentrations of zinc and selenium were determined by atomic absorption spectrophotometry at the International Institute of Tropical Agriculture, Ibadan, Nigeria. Iron was determined by a colorimetric method (Teco Diagnostics, Anaheim, USA). Total protein levels were determined by the Biuret method (23) while albumin levels were determined by the Bromo-Cresol Green dye method (24).

Statistical analysis

Statistical analysis was performed using the PAW statistic 18 package (SPSS, Chicago, USA). Results are expressed as mean ± standard deviation. Student t-test and chi-square analysis was used to analyse the data. The level of significance was set at 95% CI, where a probability value of $p < .05$ was regarded as statistically significant.

RESULTS

Table 1 shows the sociodemographic parameters of the urban and rural children. Table 2 shows a comparison of age, anthropometric indices, serum proteins and micronutrients in urban and rural children. Urban children had significantly higher weight, body mass index, total protein, albumin, selenium and frequency of meals consumed per day, but had significantly lower globulin, vitamin A, zinc and iodine and frequency of fruits consumed per week, compared to rural children. There was, however, no significant difference in mean height and iron levels between both groups. Rural children had a significantly higher frequency of thinness/wasting and stunting compared to urban children (Table 3). However, the percentage of children with normal weight was significantly higher in urban children.

Rural children had significantly higher percentage deficiencies of total protein and albumin, while urban children had significantly higher percentage deficiencies of iron and zinc (Table 4). However, neither group had vitamin A deficiency in both groups and almost all children in both the rural and urban groups were selenium deficient. There was no significant difference in the percentage deficiencies of serum iodine between both groups.

Table 5 shows the mean serum proteins and micronutrients in relation to gender in urban children. There was no significant difference in any of the parameters between both groups. However, among the rural children, boys had significantly higher weight and body mass index and significantly lower serum iron levels compared to girls (Table 6).

DISCUSSION

Globally, malnutrition is one of the most prevalent risk factors for illness and death, with pregnant women and young children being predominantly affected. In sub-Saharan Africa, malnutrition and micronutrient deficiencies remain major health burdens (25). The results of this study show that 46.4% of children in rural areas were malnourished compared to 29.6 % of children in urban areas. The prevalence rates of underweight, wasting and stunting in our study was higher than that reported by Abah *et al*. in Jos (26), but lower than that reported by Oninla *et al*. in the South Western part of Nigeria (27). However, a similar trend of rural children being more malnourished compared to urban children was observed.

Previous studies from the Southern part of Nigeria have reported an increase in obesity among children (8,11,13, 28-29). However, this trend seems to be changing in the negative direction as less than 1% obesity was recorded among the urban group and 1.8% overweight children among rural children in our study. An earlier study by Ansa *et al*., in 2001 in Calabar (the same region as that of this study) reported the prevalence of obesity as 2.3% (11). More than 46% and 22% of the rural children and 29% and 11% of the urban children were malnourished and stunted according to the WHO standards. A significantly higher percentage of the malnourished children were from the rural areas with more than a third of them suffering from hypoalbuminaemia. This is a significant pointer to the effect of the current economic crises on the health of our children which could result in an epidemic of malnutrition and its consequences on their well-being and growth.

Urban children had better body composition as they had a higher mean weight and body mass index compared to their rural counterparts. They also had a higher percentage of children in the normal weight categories. This may be attributed to the fact that many urban children have access to better nutrition due to their parents' higher socioeconomic backgrounds. Several studies have shown that urban children usually have a better nutritional status than their rural counterparts (27,30-31). This has been ascribed to the collective effect of a series of favorable socioeconomic and environmental conditions which make these children better cared for (8). This is also supported by the higher total protein and albumin levels of the urban children. In addition, access of urban children to adequate medical services and care may also be a contributing factor to their higher anthropometric indices. It is surprising, however, that rural children had higher vitamin A, zinc and iodine levels than urban children. Although urbanisation can bring about positive improvements in children's diets, it can also bring about unhealthy diet changes, such as an increase in consumption of processed foods and a decrease in fruit consumption. We observed that the urban children had a higher frequency of meals but lower frequency of fruit consumption, compared to rural children. Consumption of palm oil (which is rich in vitamin A) as well as seafoods, such as fish, crayfish and water snails (which are very rich in iodine) may be responsible for the higher vitamin A and iodine levels observed in the rural children.

Among urban children there were no differences in anthropometric indices and protein and micronutrient levels between boys and girls. However, among the rural children boys had higher weights and BMI and lower serum iron levels, compared to girls even though they were of the same age range. This may be due to fact that parents of urban children may be more enlightened about gender equality, especially when it comes to food portions. The reason for higher serum

iron levels in rural girls is not clear. Gender did not seem to have any significant effect on the frequencies of protein and micronutrient deficiencies in both urban and rural children. Virtually all children in our study were selenium deficient (99.2%.) This is in accordance with a study by Amare *et al.,* who found a 62% selenium deficiency rate (25). Low levels of selenium in soils in our region may be responsible for the selenium deficiency.

Zinc and iodine deficiencies were only observed among urban children. The prevalence of low zinc levels were found in 17% of children. However this prevalence is less than the minimum 20% prevalence set by the International Zinc consultative group as an indicator for the need for a national intervention program. The zinc deficiency observed in our study could be due to inadequate zinc intake or poor bio-absorption (32). Our findings agree with previous studies by Thurlow *et al.* (33), Folake *et al.* (34) and Dehghani, *et al.* (35) who observed the existence of zinc deficiencies among children of school age and early adolescence. However, the zero prevalence of zinc deficiency in the rural areas contrasts with a study carried out in Imo State by Onyemaobi and Onimawe who reported a high prevalence of zinc deficiency in rural areas (36). Our results show that the areas where the study was carried out is not endemic to iodine deficiency as only 2% of the urban children had iodine deficiency.

The prevalence of iron deficiency was also higher in urban children compared to their rural counterparts. The prevalence of iron deficiency recorded in our study was much lower than that reported by Onimawo *et al*. in in Southeast Nigeria (37).

We did not observe vitamin A deficiency in either the urban or rural children. This agrees with a national study by Ajaiyeoba (38) who reported that the south eastern part of the country has the lowest incidence of vitamin A deficiency. This was attributed to the fact that in the southern savannah zone palm trees are cultivated and palm oil is consumed regularly.

In conclusion, our study has shown a high prevalence of malnutrition, especially among rural children, and zinc and iron deficiency among urban children age 7 -12 years in Cross River State. There was also a worrisome high prevalence of selenium deficiency among both urban and rural children. To successfully tackle nutritional deficiencies in school-aged children, nutritional programs should be tailored to suit local needs. In urban regions, nutrition programs that encourage diet diversification strategies should be implemented to enhance micronutrient content and bioavailability. For instance, there should be an awareness about the need to improve the diet of our urban children by increasing consumption of fruits and vegetables with a view to increasing their micronutrient levels. The rural children on the other hand may benefit more from effective school feeding programs aimed at making macronutrients more available to them to reduce the rates of malnutrition. Findings from our study could aid in the planning, implementation and monitoring of regional policy initiatives for diet diversification strategies. Larger studies should be carried out to confirm the selenium deficiency observed in this study with a view to include selenium supplementation as part of micronutrient supplementation programs for the state.

Table 1. Sociodemographic characteristics of the sampled population.

Participants	Urban children n = 125	Rural children n = 125	p-value
Gender			
Females	57 (45.6)	63 (50.4)	
Males	68 (54.4)	62 (49.6)	0.448
Occupation of guardian			
Father			
Civil Servants	95 (76.0)	24 (19.2)	0.0001
Self –employed	11 (8.8)	15 (12.0)	0.408
Traders	15 (12.0)	43 (34.4)	0.0001
Farmers	0 (0.0)	40 (32.0)	0.0001
Unemployed	4 (3.2)	3 (2.4)	0.702
Mother			
Civil Servants	75 (60.0)	20 (16.0)	0.0001
Self –employed	12 (9.6)	18 (14.4)	0.244
Traders	19 (15.2)	39 (31.2)	0.003
Farmers	0 (0.0)	47 (37.6)	0.0001
Housewife	19 (15.2)	1 (0.8)	0.0001
Guardian's educational qualification			
Father			
Informal	5 (4.0)	19 (15.2)	0.003
Primary	3 (2.5)	36 (28.8)	0.0001
Secondary	17 (13.5)	50 (40.0)	0.0001
Tertiary	100 (80)	20 (16.0)	0.0001
Mother			
Informal	11 (8.8)	22 (17.6)	0.040
Primary	11 (8.8)	39 (31.2)	0.0001
Secondary	35 (28.0)	53 (42.4)	0.017
Tertiary	68 (54.4)	11 (8.8)	0.0001
Number of meals per day			
Two meals	40 (32.0)	62 (49.6)	0.005
Three meals	85 (68.0)	63 (50.4)	0.005
Fruit intake/week			
≤ 3times/week	109 (87.2)	79 (63.2)	0.0008
≥ 4 times/week	16 (12.8)	46 (36.8)	0.0001
Access to health care services			
Tertiary/secondary health centres	75 (60.0)	12 (9.6)	0.0001
Prim. healthcare centre/patent medicine shops	38 (30.4)	72 (57.6)	0.0001
Traditional medicine	12 (9.6)	41 (32.8)	0.0001

Results expressed as number (%).

Table 2. Comparison of age, anthropometric indices, serum proteins and micronutrients and food consumption in urban and rural children.

Parameters	Urban children n =125	Rural children n= 125	p-value
Age (years)	10.0 ±1.41	10.0 ±1.72	0.809
Weight (kg)	28.1 ±5.42	25.7 ±5.36	0.001
Height (m)	1.37 ±0.10	1.35 ±0.09	0.051
Body mass index (kg/m^2)	14.8 ±1.79	14.0 ±1.50	0.0001
Total protein (g/L)	77.0 ±7.21	73.5 ±10.24	0.002
Albumin (g/L)	44.1 ±4.98	37.5 ±5.58	0.0001
Globulin (g/L)	32.9 ±7.93	36.1 ±10.61	0.008
Iron (µmol/L)	19.3 ±6.52	20.6 ±5.28	0.090
Vitamin A (µmol/L)	2.88 ±0.43	3.65 ±0.63	0.0001
Selenium (µmol/L)	0.69 ±0.10	0.54 ±0.11	0.0001
Zinc (µmol/L)	12.6 ±2.32	19.3 ±4.80	0.0001
Iodine (nmol/L)	0.48 ±0.09	0.98 ±0.24	0.0001
Meals consumed per day	2.7 ±0.46	2.5 ±0.52	0.001
Fruit consumption (weekly)	2.4 ±1.44	3.3 ±1.60	0.0001

Results expressed as mean ±SD.

Table 3. Comparison of nutritional status of urban and rural children

Nutritional status	Percentage distribution n (%)		p-value
	Urban children n=125	Rural children n=125	
Thinness/wasting	36 (28.8)	56 (44.8)	0.009
Stunting	14 (11.2)	28 (22.4)	0.018
Underweight<10years#	10 (25.0)	9 (19.6)	0.550
Normal	88 (70.4)	67 (53.6)	0.006
Overweight	0 (0)	2 (1.8)	0.132
Obesity	1 (0.8)	0 (0)	0.317
Malnutrition (BMI-for-age)	37 (29.6)	58 (46.4)	0.006

n (rural) =46; n (urban) = 40

Table 4. Frequencies of protein and micronutrient deficient children in urban and rural children.

Parameters	Percentage deficiency n (%)		p-value
	Urban children n=125	Rural children n=125	
Total protein	2 (1.6)	17 (13.6)	0.0004
Albumin	8 (6.4)	42 (33.6)	0.0001
Iron	13 (10.4)	4 (3.2)	0.024
Vitamin A	0 (0)	0 (0)	-
Selenium	123 (98.4)	124 (99.2)	0.562
Zinc	17 (13.6)	0 (0)	0.0001
Iodine	2 (1.6)	0 (0)	0.157

Table 5. Mean (±SD) serum proteins and micronutrients in relation to gender in urban children.

Parameters	Boys n=68	Girls n=57	p-value
Age (years)	10.1 ±1.56	10.1 ±1.42	0.958
Weight (Kg)	27.8 ±5.88	28.4 ±4.84	0.565
Height (m)	1.37 ±0.10	1.37 ±0.09	0.992
Body mass index (Kg/m^2)	14.6 ±1.72	15.0 ±1.87	0.240
Total protein (g/L)	76.6 ±6.67	77.5 ±7.82	0.497
Albumin (g/L)	44.9 ±4.64	43.2 ±5.27	0.069
Globulin (g/L)	31.7 ±6.88	34.2 ±8.90	0.078
Iron (µmol/L)	18.4 ±6.86	20.5 ±5.95	0.073
Vitamin A (µmol/L)	2.87 ±0.32	2.89 ±0.53	0.780
Selenium (µmol/L)	0.69 ±0.12	0.68 ±0.07	0.537
Zinc (µmol/L)	12.6 ±2.26	12.6 ±2.40	0.945
Iodine (nmol/L)	0.48 ±0.09	0.48 ±0.09	0.945

Table 6. Mean serum proteins and micronutrients in relation to gender in rural children.

Parameter	Boys n= 62	Girls n =63	p-value
Age (years)	10.1 ±1.56	10.1 ±1.42	0.958
Weight (Kg)	26.9 ±5.31	24.5 ±5.18	0.012
Height (m)	1.37 ±0.10	1.33 ±0.09	0.054
Body mass index (Kg/m^2)	14.3 ±1.49	13.7 ±1.46	0.016
Total Protein (g/L)	73.7 ±9.60	73.3 ±10.91	0.811
Albumin (g/L)	37.5 ±6.15	37.5 ±5.00	0.980
Globulin (g/L)	36.3 ±9.90	35.8 ±11.33	0.808
Iron (µmol/L)	19.3 ±5.14	21.9 ±5.16	0.007
Vitamin A (µmol/L)	3.59 ±0.58	3.71 ±0.68	0.297
Selenium (µmol/L)	0.56 ±0.13	0.53 ±0.08	0.080
Zinc (µmol/L)	19.1 ±4.83	19.5 ±4.79	0.616
Iodine (nmol/L)	0.97 ±0.25	0.99 ±0.24	0.616

ACKNOWLEDGEMENTS

We would like to thank all the participants and medical personnel who took part in this study and the International Institute of Tropical Agriculture, Ibadan for use of their facilities.

AUTHOR INFORMATION

Nkechi Udo, MSc, Chief Medical Laboratory Scientist[1]
Iya E Bassey, PhD, Senior Lecturer[1]
Uwem O. Akpan[1] MSc, Lecturer[1]
Idongesit KP Isong, PhD, Lecturer[1]
Faith A Effa, PhD, Chief Medical Laboratory Scientist[2]
Augusta Ndudi, MSc, Medical Laboratory Scientist[1]

[1]Department of Medical Laboratory Science, Faculty of Allied Medical Sciences, College of Medical Sciences, University of Calabar, Calabar, Cross River State, Nigeria

[2]Department of Haematology, University of Calabar Teaching Hospital, Calabar, Cross River State, Nigeria

Corresponding author: Dr. Iya Eze Bassey.
Email: iyantui@yahoo.com

REFERENCES

1. National Academies of Sciences, Engineering, and Medicine; Health and Medicine Division; Board on Global Health; Committee on Global Health and the Future of the United States. Global Health and the Future Role of the United States. Washington (DC): National Academies Press (US); 2017 May 15. 5, Investing in Women's and Children's Health.Available from: https://www.ncbi.nlm.nih.gov/books/NBK458467/.
2. World Health Organization. Field guide on rapid nutritional assessment in emergencies. Alexandria, Egypt: Regional Office for the Eastern Mediterranean; 1995. Available from http://applications.emro.who.int/dsaf/dsa55.pdf.
3. Institute of Medicine (US) Committee on Assuring the Health of the Public in the 21st Century. The Future of the Public's Health in the 21st Century. Washington (DC): National Academies Press (US); 2002. 2, Understanding Population Health and Its Determinants. Available from: https://www.ncbi.nlm.nih.gov/books/NBK221225/.
4. National Population Commission (NPC) (Nigeria) & ICF International. NDHS. 2014. Abuja, Nigeria: National Population Commission and ICF International, 2013.

5. Herrador Z, Sordo L, Gadisa E, Buño A, Gómez-Rioja R, Iturzaeta JM, et al. Micronutrient deficiencies and related factors in school-aged children in Ethiopia: a cross-sectional study in Libo Kemkem and Fogera districts, Amhara Regional State. *PLoS One* 2014; 9(12) :e112858.
6. Adelakan DA. Multiple micronutrient deficiencies in developing countries. *Nutrition.* 2003; 19(5):473-474.
7. Uchendu FN. Micronutrient malnutrition, a tragedy to childhood growth and education. *Glob J Med Res* 2011; 11 (1).
8. Ene-Obong H, Ibeanu V, Onuoha N, Ejekwu A. Prevalence of overweight, obesity, and thinness among urban school-aged children and adolescents in southern Nigeria. *Food Nutr Bull* 2012; 33(4): 242 – 250.
9. Adamu A, Jiya NM, Ahmed H, Ibitoye PK, Ugege MO, Sani UM, et al. Prevalence of Vitamin A Deficiency among Malnourished Children in Usmanu Danfodiyo University Teaching Hospital, Sokoto, Northwestern Nigeria. *Pak J Nutr* 2016; 15(9): 821-828.
10. Abubakar N, Atiku MK, Alhassan AJ, Mohammed IY, Garba RM, Gwarzo GD. An assessment of micronutrient deficiency: a comparative study of children with protein-energy malnutrition and apparently healthy controls in Kano, Northern Nigeria. *Trop J Med Res* 2017; 20: 61-65.
11. Ansa VO, Odigwe CO, Anah MU. Profile of body mass index and obesity in Nigerian children and adolescents. *Niger J Med*; 2001; 10 (2): 78-80.
12. Usoro CAO, Udoh AE, Ntui I. Biochemical changes in serum of protein-energy malnourished children following dietary protein repletion. *Glob J Pure Appl Sci* 2003; 9(1): 43 – 46.
13. Anah MU, Ikpeme OE, Etuk IS, Eyong KI, Bassey AE. Nutrition and health status of preschool children of rubber plantation workers in Calabar, Nigeria. *Mary Slessor J Med* 2009; 9: 2.
14. Kish L. Survey Sampling. Wiley, New York, NY, USA, 1965.
15. World Health Organization. Growth reference data for 5-19 years. WHO reference, 2007. Available from http:// www.who.int/growthref/en/ . Last assessed 23 January 2018.
16. Sauberlich HE. Laboratory Tests for the Assessment of Nutritional Status. Boca Raton: CRC Press, USA, 1999.
17. Jackson AA. Identifying children at risk of malnutrition. *Nutr J* 2018; 17(1): 84.
18. Nhien NV, Khan NC, Yabutani T, Ninh NX, Chung le TK, Motonaka J, et al. Relationship of low serum selenium to anemia among primary school children living in rural Vietnam. *J Nutr Sci Vitaminol (Tokyo)* 2008; 54: 454–459.
19. Campa A. Cutoff point for selenium deficiency. *Eur J Clin Nutr* 2012; 66: 1277 [Letter].
20. Santoro JD, Nespor C, Poole RL, Kerner JA Jr. Iodine supplementation for pediatric patients receiving long-term parenteral nutrition. *Nutr Clin Pract* 2016; 31: 245-249.
21. Centers for Disease Control and Prevention. Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population. National Center for Environmental Health; Atlanta, GA, USA: April 2012. (accessed on 22 June 2017). http://www.cdc.gov/ nutritionreport/pdf/Nutrition_Book_complete508_final.pdf.
22. Pino S, Fang SL, Braverman LE. Ammonium persulfate: a new and safe method for measuring urinary iodine by ammonium persulfate oxidation. *Exp Clin Endocrinol Diabetes* 1998; 106(Suppl 3): S22–S27.
23. Doumas BT, Bayse D, Carter RJ, Peters T Jr, Schaffer R. A candidate reference method for determination of total protein in serum. 1. Development and validation. *Clin Chem* 1981; 27:1642-1650.
24. Doumas BT, Watson WA, Biggs HG. Albumin standards and the measurement of serum albumin with bromcresol green. *Clin Chim Acta.* 1971; 31(1):87-96.
25. Amare B, Moges B, Fantahun B, Tafess K, Woldeyohannes D, Yismaw G, et al. Micronutrient levels and nutritional status of school children living in Northwest Ethiopia. *Nutr J* 2012; 11:108.
26. Abah RO, Okolo SN, John C, Ochoga MO, Adah RO. Nutritional status of schoolchildren in Jos East Local Government Area of Plateau State, North Central Nigeria. *J Med Trop* 2017; 19:56-59.
27. Oninia SO, Owa JA, Onayade AA, Taiwo O. Comparative study of nutritional status of urban and rural Nigerian school children. *J Trop Pediatr* 2006; 53(1): 39 - 43
28. Opara DC, Ikpeme EE, Ekanem US. Prevalence of stunting, underweight and obesity in school-aged children in Uyo, Nigeria. *Pak J Nutr* 2010; 9: 459-466.
29. Nwaiwu O, Ibe BC. Body mass index of children aged 2 to 15 years in Enugu Nigeria. *Niger J Paed* 2014; 41 (3): 194 –198.
30. Anwer I, Awan JA. Nutritional status of rural and urban school children in Faisalabad District, Pakistan. *Rural Remote Health* 2003; 3: 130.
31. Smith LC, Ruel MT, Ndiaye A. Why is child malnutrition lower in urban than in rural areas? Evidence from 36 developing countries. *World Dev* 2005; 33: 1285–1305.
32. Kawade R. Zinc status and its association with the health of adolescents: a review of studies in India. *Glob Health Action* 2012; 5: 7353.
33. Thurlow RA, Winichagoon P, Pongcharoen T, Gowachirapant S, Boonpraderm A, Manger MS, et al. Risk of zinc, iodine and other micronutrient deficiencies among school children in North-East Thailand. *Eur J Clin Nutr* 2006; 60: 623–632.
34. Samuel FO, Egal AA, Oldewage-Theron WH, Napier CE, Venter CS. Prevalence of zinc deficiency among primary school children in a poor peri-urban informal settlement in South Africa. *Health SA Gesondheid* 2010; 15(1): 1–6.
35. Dehghani SM, Katibeh P, Haghighat M, Moravej H, Asadi S. Prevalence of zinc deficiency in 18 years old children in Shiraz-Iran. *Iran Red Crescent Med J* 2011; 13(1): 4–8.
36. Onyemaobi GA, Onimawo IA. Zinc status of under-five children in rural and urban Imo State, Nigeria. *J Basic Appl Sci Res* 2011; 1: 451-45.
37. Onimawo IA, Ukegbu PO, Asumugha VU, Anyika JU, Okudu H, Echendu CA, et al. Assessment of anaemia and iron status of school age children (aged 7-12 years) in rural communities of Abia state, Nigeria. *Afr J Food Agric Nutr Dev* 2010; 10(5): 2570-2586.
38. Ajaiyeoba AI. Vitamin A deficiency in Nigerian children. *Afr J Biomed Res* 2001; 4: 107 -110.

ORIGINAL ARTICLE

Diagnostic accuracy of the serum biomarker S100A8/A9 and Alvarado score in the diagnosis of appendicitis

Hassan Motamed, Meysam Moezzi, Mohamad Ali Fahimi, Samaneh Porozan, Nastaran Ranjbari and Mohammad Ali Ghiass

ABSTRACT

Introduction: Appendicitis is one of the most common causes of patients referring to emergency departments with abdominal pain. The aim of the study was to investigate the diagnostic accuracy of the serum biomarker S100A8/A9 in diagnosing appendicitis.

Methods: In a cross-sectional study patients suspected of appendicitis admitted to the emergency department were selected using a non-probability consecutive sampling technique. After an initial visit by an emergency medicine specialist and provision of the initial evaluation, if the diagnosis of acute appendicitis in the patient was suspected, blood samples and the required tests were taken to determine the serum level of the biomarker S100A8/A9. Moreover, the Alvarado score of the patient was determined. Based on the clinical findings, ultrasonography was performed and, if necessary, a surgeon was consulted.

Results: 166 patients with suspected appendicitis were entered into the study. The mean age of patients was 12 ± 5.4 years. The average elapsed time from onset of pain to time of patient referral was 2.5 ± 1.1 hours (between 1 to 4 hours). Sensitivity, specificity, positive predictive value and negative predictive value of serum S100A8/A9 were 61.9%, 40.2%, 39%, and 63.6 %, respectively. The area under the ROC curve for S100A8/A9 was 0.5 ± 0.5.

Conclusion: The study results indicate that serum levels of S100A8/A9 have a low sensitivity and specificity; therefore, they have no diagnostic value in appendicitis.

Keywords: biomarker, S100A8/A9, appendicitis, sensitivity, specificity.

N Z J Med Lab Sci 2020; 74: 135-139.

INTRODUCTION

Acute appendicitis is one of the most common causes of patients' referral to an emergency department with abdominal pain (1). It is reported that up to 7% of people experience acute appendicitis during their lifetime, and it often occurs between the ages of 10 and 30 years (2). Despite the advances made in diagnostic procedures, the diagnosis of acute appendicitis is still a surgical problem and the negative appendectomy rate is between 8-12% in men and 25-45% in women (3-5). At present, the diagnosis of appendicitis is mainly clinical, and laboratory tests such as white blood cell counts and WBC differentials help in the diagnosis (6). The timely and correct diagnosis of appendicitis is necessary to reduce complications, such as a ruptured appendix. Moreover, a negative appendectomy has surgical and anesthesia complications similar to a positive appendectomy, including postoperative infections, intestinal obstruction due to adhesions, and the probability of infertility in young women (7,8).

Although the Alvarado score is commonly used, it has many drawbacks (1). This ten points score has six clinical and two laboratory items (9). Including the fact that the framework of the Alvarado scoring system is based on the examination of patients with suspected appendicitis who have undergone surgery. Moreover, there is no place for biomarkers in this scoring system, while the majority of studies conducted on the role of biomarkers in appendicitis state that they play a very important role in the diagnosis of acute appendicitis (10). There is growing evidence of a close connection between immune responses and the nervous system, especially in the intestines, and it is believed that the nervous system plays an important role in regulating the immune response in the gastrointestinal tract (11-13). The evidence includes the presence of neuropeptide receptors on immune cells in the gastrointestinal tract (14).

S100A8, also known as calgranulin A, (myeloid-related protein 8 [Mrp8]) and S100A9, also known as calgranulin B (myeloid-related protein 14 [Mrp14]) are intracellular calcium-binding proteins which are the keys to calcium signaling transmission during inflammation (15). These two independent proteins express a specific tissue pattern and are easily combined in dimer form known as S100A8/A9 or calprotectin (6). S100A8/A9 is mainly expressed in neutrophils, monocytes, some epithelial cells and keratinocytes in tissue inflammation, while it is not generally expressed in tissue macrophages or lymphocytes (15,16). The expression of S100A8 and S100A9 at these types of inflammatory sites is well known and the evidence of S100A8/A9 can be indicative of inflammatory conditions (15,17).

Given the limited studies on S100A8/A9 and because no research has been conducted on this topic in Iran, the aim of our study was to investigate the diagnostic accuracy of this biomarker and the Alvarado score in the diagnosis of appendicitis.

METHODS

Study design

This study was a cross-sectional design in an established time frame from the beginning until the end of 2017. The study subjects were all patients suspected of appendicitis who were admitted to the emergency department of Imam Khomeini Hospital were selected for the study using non-probability consecutive sampling technique. Ethical approval was by the Ethics Committee in Ahvaz, Iran (IR.AJUMS.REC.1396.377). Inclusion criteria comprised suspected appendicitis and an age between 2 and 18 years. Exclusion criteria comprised aged below 2 years or above 18 years, pregnancy, a history of a previous appendectomy, inflammatory diseases, active cancers, abdominal trauma, or surgical or invasive abdominal

procedure in the preceding seven days, use of corticosteroids in the preceding 14 days, receiving chemotherapy or immunosuppressive drugs in the preceding 29 days, the presence of a urinary tract infection or urinary tract pathology (WBC>20 x 10^9/L and RBC>30 x 10^9/L in urine analysis).

Patients who came to the emergency department with abdominal pain, were entered into the study subsequent to an initial visit by an emergency medicine specialist and the provision of the initial evaluation, meeting the inclusion criteria, and after providing informed consent to participate. If acute appendicitis was suspected, blood samples and the required tests were taken before surgery to determine the serum levels of S100A8/A9. Moreover, an Alvarado score was determined for the patient. Based on the patient's clinical findings, ultrasonography was performed and, if necessary, a surgeon was consulted.

Obtaining the medical history of all patients, performing physical examinations, and performing assessments for the Alvarado scoring system were accomplished by the emergency medicine specialist. Symptoms included migratory pain, anorexia, nausea and vomiting, right abdominal quadrant tenderness, rebound tenderness, temperature rise, leukocytosis (> 11,000/mm^3), and shift to left.

Patients were divided into three groups according to their Alvarado scores: low-risk (scores < 4), moderate-risk (scores 4, 5 or 6), and high-risk (scores > 6). Ultrasonography was performed for patients if needed. Blood samples were taken from all patients, and serum levels of S100A8/A9 were measured.

Serum levels of S100A8/A9 was measured by a sandwich enzyme-linked immunosorbent assay (Aspen Bio Pharma, Inc. Castle Rock, Co) according to the manufacturer's protocol. Patients diagnosed with appendicitis underwent an appendectomy. The appendix was sent to the pathology laboratory for histology. Patients received follow-up telephone calls 14 days after discharge. Results were also recorded for patients who were ultimately given another diagnosis. The COSMIN risk of bias checklist was used to determine the methodological consistency of the study (19) while the Standards for Reporting of Diagnostic Accuracy Studies (STARD) checklist was used to report on the accuracy of a diagnostic test (18).

Sample size

Considering the sensitivity of 77% for biomarkers in the diagnosis of appendicitis and taking into account the error of the first type 5% and accuracy (d = 0.77), a minimum sample size of 139 patients was estimated to be required (20).

Data analysis

Quantitative variables are presented as mean ± standard deviation, and qualitative variables as number with percentage. The T-test or Mann Whitney U test as well as the Chi-squared test was used for analysing the data. To assess the diagnostic value of biomarkers, the sensitivity and specificity were determined. The ROC curve was also used to determine the cut-off point and diagnostic accuracy (21). The area under the ROC curve was calculated to determine the predictive power of S100A8/A9. The McNemar's test was used to compare the results of two diagnostic tests. A significance level at confidence interval (CI) of 95% and p-value of <0.05 was approved and all data analysis was performed using SPSS 22 (IBM, Chicago).

RESULTS

166 patients with suspected appendicitis admitted to the emergency department were investigated. The mean age of the patients participating in the study was 12 ± 5.4 years. Ninety-one patients (54.8%) were male and 75 patients (45.2%) were female. The average elapsed time from onset of pain to patient attendance was 2.5 ± 1.1 hours (between 1 to 4 hours) (Table 1).

Assessment of the Alvarado score showed migratory pain in 62 patients in total and in 40 patients with appendicitis. Moreover, anorexia was observed in 145 patients in total and in 56 patients with appendicitis; nausea and vomiting was seen in 134 patients in total and in 51 patients with appendicitis; fever was seen in 77 patients in total and in 35 patients with appendicitis; rebound tenderness was observed in 107 patients in total and in 54 patients with appendicitis; and right abdominal quadrant tenderness was observed in all study participants (Table 2).

The white blood cell count was higher than 10 x10^9/L in 120 study participants in total and in patients with appendicitis; neutrophils were higher than 75% in 109 study participants and among patients with appendicitis (Table 3). Assessment of the Alvarado score showed a score between 0 and 3 for three study participants, none of whom had appendicitis; 50 participants scored 4 to 6 of whom eight had appendicitis; 112 participants scored greater than 6 of whom 54 had appendicitis. Therefore, the sensitivity, specificity, positive predictive value and negative predictive value of the Alvarado score in diagnosing appendicitis were 87.09%, 43.68%, 40.17% and 84.9%, respectively (Table 3).

In total, 114 patients had normal results on ultrasonography, 17 of whom had appendicitis; 51 patients had abnormal results, 45 of whom had appendicitis. Based on these results, the sensitivity, specificity, positive predictive value, and negative predictive value of ultrasonography in diagnosing appendicitis was 72.6%, 94.1%, 88.2% and 85 %, respectively (Table 3). Furthermore, the pathology of patients with appendicitis showed that 25 cases had acute appendicitis with peri-appendicitis, 17 cases had acute suppurative appendicitis with peri-appendicitis, and 21 cases had gangrenous perforated appendicitis.

The ROC test was used to determine the diagnostic power of serum S100A8/A9 in the diagnosis of appendicitis. The AUROC value for this prediction criterion lies in the range of 0.5 ± 0.5. Picking a probability model threshold of 0.75 for the biomarker, the sensitivity, specificity, positive predictive value, and negative predictive value were computed to be 61.9% and 40.2%, 39% and 63.6 %, respectively (Figure 1).

Table 1. Demographics of patients with and without appendicitis.

Characteristics	Appendicitis group N=63	Without appendicitis group N=103	Both groups N=166	p*
Age (years)	12.7 ±5.9	11.5 ± 5	12.0 ± 5.4	0.149
Pain onset (hours)	2.5 ±1.1	2.5 ±1.2	2.5 ±1.1	0.951

*Independent t-test. Results are mean ± standard deviation.

Table 2. Symptoms and Alvarado score in patients with and without appendicitis.

Characteristics	Type	Appendicitis group (N=63)	Without appendicitis group (N=103)	Both groups (N=166)	P*
Gender. N (%)	Male	45 (71.4%)	46 (44.7%)	91 (54.8%)	0.001
	Female	18 (28.6%)	57 (55.3%)	75 (45.2%)	
Migratory pain. N (%)	Positive	40 (64.5%)	22 (21.4%)	62 (37.6%)	<0.001
	Negative	22 (35.5%)	81 (78.6%)	103 (62.4%)	
Anorexia. N (%)	Positive	56 (90.3%)	89 (86.4%)	145 (87.9%)	0.623
	Negative	6 (9.7%)	14 (13.6%)	20 (12.1%)	
Nausea and vomiting. N (%)	Positive	51 (82.3%)	83 (80.6%)	134 (81.2%)	0.84
	Negative	11 (17.7%)	20 (19.4%)	31 (18.8%)	
Fever. N (%)	Positive	35 (56.5%)	42 (40.8%)	77 (46.7%)	0.055
	Negative	27 (43.5%)	61 (59.2%)	88 (53.3%)	
Rebound tenderness. N (%)	Positive	54 (87.1%)	53 (51.5%)	107 (64.8%)	<0.001
	Negative	8 (12.9%)	50 (48.5%)	58 (35.2%)	
RAQ tenderness. N (%)	Positive	62 (98.4%)	100 (97.1%)	165 (99.4%)	<0.001
	Negative	0	0	0	
Alvarado score. N (%)	Low risk	0	3 (2.9%)	3 (1.8%)	<0.001
	Moderate risk	8 (12.9%)	42 (40.8%)	50 (30.6%)	
	High risk	54 (48.2%)	58 (56.3%)	112 (67.6%)	

*Chi-square test. Results are number with percentage. RAQ = right abdominal quadrant.

Table 3. Characteristics of tests in patients with and without appendicitis.

Characteristics	Type	Appendicitis group (N=63)	Without appendicitis group (N=103)	Total (N=166)	P*
Leukocytosis.N (%)	Yes	56 (90.3%)	64 (62.1%)	120 (72.7%)	<0.001
	No	6 (9.7%)	39 (37.9%)	45 (27.3%)	
PMN>75%. N (%)	Yes	54 (87.1%)	55 (46.6%)	109 (66.1%)	<0.001
	No	8 (12.9%)	48 (53.4%)	56 (33.9%)	
Ultrasonography. N (%)	Appendicitis	45 (72.6%)	97 (94.2%)	51 (30.9%)	<0.001
	Normal	17 (27.4%)	6 (5.8%)	114 (69.1%)	
WBC $x10^9$/L. Mean ± SD		14.83 ± 3.76	12.09 ±7.75	13.12 ± 6.66	0.01
PMN $x10^9$/L. Mean± SD		0.082 ±0.008	0.073 ±0.012	0.077 ±0.012	<0.001
Biomarker (NM/ML). Mean± SD		1.7 ± 3	1.8 ±3.3	1.8 ±3	0.831

*Chi-square test.

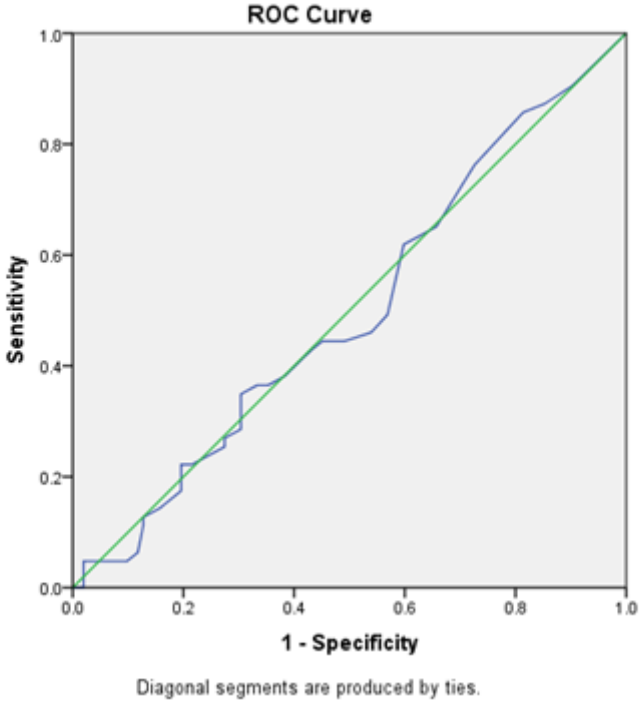


Figure 1. ROC curve of serum S100A8/A9 in the diagnosis of appendicitis.

DISCUSSION

In the present study, the biomarker S100A8/A9, which has been reported to have a more topical inflammatory response and is directly related to increased neutrophil activity, was investigated. The results showed that the serum levels of this protein differ in patients with appendicitis compared with patients with abdominal pain who did not have appendicitis. In the present study, the area under the ROC curve was 0.46±0.498 for the biomarker indicating that S100A8/A9 has no role in the diagnosis of appendicitis because of its low predictive value.

Bealer *et al*., investigating the relationship between serum levels of S100A8/A9 and appendicitis, showed that the sensitivity and specificity of S100A8/A9 in the diagnosis of acute appendicitis was 93% and 54%, respectively, while the positive and negative predictive values were 37% and 96%, respectively (22). The authors concluded that this biomarker could be used in the diagnosis of appendicitis along with other methods. In our study, sensitivity was 61.9%, specificity 40.2%, positive predictive value 39%, and negative predictive value 63.63%, thus our results are inconsistent with the study results of Bealer *et al*.

Mills *et al.* investigated the relationship between serum biomarkers S100A8/A9 and pain in the RLQ region (23). They showed that the sensitivity and specificity of S100A8/A9 in the diagnosis of acute appendicitis was 96% and 16% %, respectively. Negative LR and positive LR of serum biomarkers S100A8/A9 were 0.24 and 1.14, respectively. In the same study, the researchers ultimately concluded that serum biomarkers S100A8/A9 had a high sensitivity, but low specificity; therefore, they could be used along with other tests.

Some studies have reported different results, including the study by Kentsis *et al*, in which, the authors evaluated the validity and value of three urinary markers, including S100A8, in 67 patients with an average age of 11 years, 37% of whom had appendicitis. They found that S100A8 had an area under the ROC curve of 0.84 (0.72 to 0.95). They concluded that the measurement of S100A8 could increase the diagnostic accuracy of appendicitis. Their study differed from our study in that S100-A8 levels were measured in urine, while serum levels of S100A8/A9 were measured in our current study.

Thuijls *et al*. compared 51 patients who had undergone surgery for appendicitis with 21 healthy individuals (25). They reported that the mean serum levels of S100A8 were significantly higher in the appendicitis group than in the control group. The results of this study are also inconsistent with the results of our study. Cikot *et al*. examined the use of S100 in distinguishing uncomplicated from complicated appendicitis (26). They studied 23 patients with complicated appendicitis and 66 patients with uncomplicated appendicitis and showed that the levels of this biomarker were significantly higher in the complicated group than in the uncomplicated group.

The contradictory results between our study and some other studies may have arisen from the type of patients considered as a control group. In our study, participants in the control group were not healthy and suffered from abdominal pain. Given that the expression and secretion of the S100 protein are affected by many different factors, including inflammatory and biological processes, some other factors may increase this protein level in the control group and lead to insignificant results (27). Moreover, the measurement of fecal S100 protein levels might be a better reflector of the typical responses resulting from appendicitis. Therefore, it is suggested that this method also be considered in subsequent studies.

In our study, the sensitivity of ultrasonography in diagnosing appendicitis was 72.58%. The sensitivity of ultrasonography in the diagnosis of acute appendicitis has been reported to be between 87% and 96% in other studies (28-30). Ultrasonography is a method often used to diagnose acute appendicitis, especially in women. Therefore, it is not a suitable criterion for appendectomy. Moreover, patients should not be discharged because of normal results on ultrasonography.

A limitation of our study was that the sample size was small and the study was retrospective in nature. In conclusion, the serum biomarker S100A8/A9 has low sensitivity and specificity; therefore, along with other tests, their routine use in the diagnosis of appendicitis is not recommended. Our study showed that the roles of clinical examination and laboratory findings in the diagnosis of acute appendicitis were more significant than ultrasonography, especially in healthcare centers where ultrasonography is not available. Moreover, diagnostic accuracy becomes very high if both methods are used.

ACKNOWLEDGMENTS

This study was financially supported by the Office of the Vice-chancellor for Technology and Research Development of Jundishapur Medical Sciences University, Ahvaz, Southwest Iran as part of Ali Ghiass's thesis under the research code U-96071. We gratefully acknowledge the dedicated efforts of the investigators, the coordinators, the volunteer patients who participated in this study, and the Clinical Research Development Units of Ahvaz Golestan Hospital.

Funding source, financial disclosures
Ahvaz Jundishapur University of Medical Sciences number: U-96071.

AUTHOR INFORMATION

Hassan Motamed[1]
Meysam Moezzi[1]
Mohamad Ali Fahimi[1]
Samaneh Porozan[1]
Nastaran Ranjbari[2]
Mohammad Ali Ghiass[1]

[1]Emergency Medicine Department, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Department of Pathology, Imam Khomeini Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Corresponding Author
meisam.moezzi@yahoo.com

REFERENCES

1. Douglas CD, Macpherson NE, Davidson PM, Gani JS. Randomised controlled trial of ultrasonography in diagnosis of acute appendicitis, incorporating the Alvarado score. *BMJ* 2000; 321: 919-922.
2. Rosengren D, Brown AF, Chu K. Radiological imaging to improve the emergency department diagnosis of acute appen dicitis. *Emerg Med Australas* 2004; 16: 410-416.
3. Raman SS, Lu DS, Kadell BM, et al. Accuracy of nonfocused helical CT for the diagnosis of acute appendicitis: a 5-year review. *AJR Am J Roentgenol* 2002; 178: 1319-1325.
4. Wong SK, Chan LP, Yeo A. Helical CT imaging of clinically suspected appendicitis: correlation of CT and histological findings. *Clin Radiol* 2002; 57: 741-745.
5. Fuchs JR, Schlamberg JS, Shortsleeve MJ, Schuler JG. Impact of abdominal CT imaging on the management of appendicitis: an update. *J Surg Res* 2002; 106: 131-136.
6. Kaiser S, Jorulf H, Söderman E, Frenckner B. Impact of radiologic imaging on the surgical decision-making process in suspected appendicitis in children. *Acad Radiol* 2004; 11: 971-979.
7. Alvarado A. A practical score for the early diagnosis of acute appendicitis. *Ann Emerg Med* 1986; 15: 557-564.
8. Kang HJ, Kang H, Kim B, et al. Evaluation of the diagnostic performance of a decision tree model in suspected acute appendicitis with equivocal preoperative computed tomography findings compared with Alvarado, Eskelinen, and adult appendicitis scores: A STARD compliant article. *Medicine (Baltimore)* 2019; 98: e17368.
9. Ohle R, O'Reilly F, O'Brien KK, et al. The Alvarado score for predicting acute appendicitis: a systematic review. *BMC Med* 2011; 9: 139.
10. Andersson RE. Meta-analysis of the clinical and laboratory diagnosis of appendicitis. *Br J Surg* 2004; 91: 28-37.
11. Coskun N, Sindel M, Elpek GO. Mast cell density, neuronal hypertrophy and nerve growth factor expression in patients with acute appendicitis. *Folia Morphol (Warsz)* 2002; 61: 237- 243.
12. Mysorekar VV, Chanda S, Dandeka CP. Mast cells in surgically resected appendices. *Indian J Pathol Microbiol* 2006; 49: 229-233.
13. Coşkun N, Indel SM, Elpek GO. Mast cell density and neuronal hypertrophy in patients with acute appendicitis. *Turk J Gastroenterol* 2003; 14: 54-58.
14. Xiong S, Puri P, Nemeth L, et al. Neuronal hypertrophy in acute appendicitis. *Arch Pathol Lab Med* 2000; 124: 1429-1433.
15. Passey RJ, Xu K, Hume DA, Geczy CL. S100A8: emerging functions and regulation. *J Leukoc Biol* 1999; 66: 549-556.
16. Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. *J Leukoc Biol* 1993; 53: 197-204.
17. Stríz I, Trebichavský I. Calprotectin - a pleiotropic molecule in acute and chronic inflammation. *Physiol Res* 2004; 53: 245-253.
18. Cohen JF, Korevaar DA, Altman DG, et al. STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration. *BMJ Open* 2016; 6: e012799.
19. Terwee CB, Prinsen CAC, Chiarotto A, et al. COSMIN methodology for evaluating the content validity of patient-reported outcome measures: a Delphi study. *Qual Life Res* 2018; 27: 1159-1170.
20. Schellekens DH, Hulsewé KW, van Acker BA, et al. Evaluation of the diagnostic accuracy of plasma markers for early diagnosis in patients suspected for acute appendicitis. *Acad Emerg Med* 2013; 20: 703-710.
21. Hoo ZH, Candlish J, Teare D. What is an ROC curve? *Emerg Med J* 2017; 34: 357-359.
22. Nadoushan MR, Moradi M, Akbari HM, Fallah N. The comparison of nerve fibers, schwann cells and mast cells in acute appendicitis with normal and non inflamed appendix with clinical symptoms. *J Gorgan Univ Med Sci* 2008; 9: 47-50.
23. Bealer JF, Colgin M. S100A8/A9: a potential new diagnostic aid for acute appendicitis. *Acad Emerg Med* 2010; 17: 333-336.
24. Kentsis A, Lin YY, Kurek K, et al. Discovery and validation of urine markers of acute pediatric appendicitis using high-accuracy mass spectrometry. *Ann Emerg Med* 2010; 55: 62-70. e4.
25. Thuijls G, Derikx JP, Prakken FJ, et al. A pilot study on potential new plasma markers for diagnosis of acute appendicitis. *Am J Emerg Med* 2011; 29: 256-60.
26. Cikot M, Peker KD, Bozkurt MA, et al. Plasma calprotectin level: usage in distinction of uncomplicated from complicated acute appendicitis. *World J Emerg Surg* 2016; 11: 7.
27. Dhas DBB, Bhat BV, Gane DB. Role of calprotectin in infection and inflammation. *Curr Pediatr Re*s 2012; 16: 83-94.
28. Birnbaum BA, Wilson SR. Appendicitis at the millennium. *Radiology* 2000;215: 337-348.
29. Paulson EK, Kalady MF, Pappas TN. Suspected appendicitis. *New Engl J Med* 2003; 348: 236-242.
30. Yacoe ME, Jeffrey Jr RB. Sonography of appendicitis and diverticulitis. *Radiol Clin North Am* 1994; 32: 899-912.

CASE STUDY

Production and disappearance of multiple red cell alloantibodies in a patient with pure red cell aplasia: a case study

Bhavisha Solanki, Raewyn Cameron, Diane Matheson, William Perry and Holly Perry

ABSTRACT

Alloimmunisation against red cell antigens poses a challenge to blood transfusion. This case study illustrates the difficulty in providing compatible red cells to a patient with multiple, but non-persistent alloantibodies. The patient, who was transfusion-dependent due to pure red cell aplasia, produced multiple alloantibodies against antigens of three blood group systems. The persistence of the alloantibodies in this patient was much shorter than expected.

Key words: transfusion, alloimmunisation, multiple alloantibodies, non-persistent alloantibodies, immune responder.

N Z J Med Lab Sci 2020; 74: 140-143.

INTRODUCTION

Human red blood cells (RBC) express multiple antigens across 38 blood group systems (1). When the immune system encounters foreign antigens it may produce the corresponding specific alloantibody. Production of alloantibodies occurs in approximately 2%-6% of patients exposed to foreign red cell antigens through blood transfusion or pregnancy (2).

An antibody screen detects the presence of antibodies of clinical significance outside the ABO system. Patient plasma is tested against a set of three reagent red cells that between them express the major red cell antigens of the Rh, Kell, Duffy, Kidd, Lewis, P, MNS and Lutheran blood group systems (3). A positive reaction with one or more screening cells signals the presence of clinically significant antibodies, after which antibody identification is performed using an extended panel of cells. The pattern of positive and negative reactions obtained with the patient plasma and the panel cells is matched to the pattern of antigenic profile of the panel cells to identify the specificity of the antibody or antibodies. When the reaction pattern does not match any antigenic profile, the antibody is called an antibody of undetermined specificity (AUS) (4). Liu and Grossman (4) explain that an AUS finding may be insignificant, but that a portion may indicate alloantibodies in early development or alloantibodies to low incidence antigens.

Patients with a negative alloantibody screen require red blood cells compatible with only their ABO and Rh(D) groups. Those with alloantibodies to blood group antigens require RBC that do not express the corresponding antigen(s). A small percentage of patients may produce multiple alloantibodies, especially those who have been multi-transfused over time (5). This complicates the process of providing compatible RBC (5,6), as there may be only small numbers of blood donors lacking the corresponding RBC antigens. Many people may never make red cell alloantibodies despite receiving multiple transfusions, whereas some may be immunised after the transfusion of a single unit of RBC expressing foreign antigens (2). Alloimmunisation to red cell antigens following transfusion depends on both genetic and acquired patient factors, as well as the dose and immunogenicity of the antigens (6).

The purpose of this case study is to illustrate the challenges associated with the provision of RBC for a patient with multiple, but non-persistent, red cell alloantibodies.

CASE STUDY

A 36-year-old woman was diagnosed with pure red cell aplasia, a syndrome that results in a marked decrease or complete absence of red cell production from the bone marrow (7). Patients with this condition are dependent on RBC transfusion to maintain haemoglobin levels compatible with life.

The patient also presented with insulin-dependent diabetes mellitus and end stage renal disease. She had Hepatitis C, was a cigarette smoker and had a history of recreational drug use. Her long-term medications included insulin and a glucose control drug, anti-convulsants, vitamin D and calcium supplements, antacid and proton pump inhibitors, doxazosin (for blood pressure management) and laxatives. Methadone and the antibiotic vancomycin were prescribed as needed.

Due to her pure red cell aplasia, the patient's haemoglobin levels were persistently low and the patient was transfusion-dependent for six years (2012-2019), receiving a total of 128 RBC units from multiple blood donors over this period (Table 1).

Blood grouping was performed on an automated Ortho platform utilizing bead-based column agglutination technology. The patient's blood group was A Rh(D) positive. Her extended phenotype was R_1r (DCce), K negative, Fy(a+b+), Jk(a+b-), S+s+ (Table 2). Over the six-year period she produced alloantibodies with the specificities of anti-E in the Rh system, anti-Jk^b in the Kidd system and multiple Kell system antibodies (anti-K, anti-Kp^a and anti-Js^a). There was also the presence of a transient autoantibody detected during antibody screening (Table 1).

The patient's direct antiglobulin test (DAT) was tested on five occasions across the six-year period and was positive whenever tested, indicating the presence of immunoglobulin and complement on the patient's RBC (Table 3). An eluate performed on the DAT positive cells on 31st October 2013 showed no pattern of antibody specificity.

When transfused, the patient received group A and O Rh(D) positive and Rh(D) negative RBC. As she produced alloantibodies she received RBC negative for the corresponding antigens, compatible by indirect antiglobulin crossmatch in order to avoid haemolytic transfusion reactions (HTR).

The patient first presented with a positive red cell antibody screen in early July 2013 after being transfused with three units of RBC in December 2012. As Table 1 shows, she went on to produce many more alloantibodies. In fact, she progressively produced antibodies for each antigen that she

lacked across the Rh, Kidd and Kell systems. The description of AUS (Table 1) may in retrospect be attributable to early production of some of these alloantibodies (April 2014 and late July and August 2016). The AUS produced late in the six-year period (November 2018 to February 2019) may be attributable to alloantibodies to low incidence antigens. The frequency of RBC transfusion decreased from 2018 (Table 1) and the patient died of renal failure in early 2019.

DISCUSSION

In this case study we report a transfusion-dependent patient with pure red cell aplasia who formed red cell alloantibodies to multiple antigens that she lacked in the Rh, Kidd and Kell blood group systems, classifying her as a strong "immune responder" (6). This led to difficulties in the provision of compatible RBC. Alloantibodies became undetectable over time and whilst this can be the case for several alloantibody specificities (2, 8-10), this patient's alloantibodies declined more quickly than expected (10).

Most humans do not produce red cell alloantibodies when transfused (2) even though they are exposed to non-self antigens. Almost every transfusion will expose the recipient to foreign antigens and people who do not produce antibodies are termed "non-responders"(6). The patient presented in this case study was a strong responder.

People who have formed alloantibodies as a result of transfusion are more likely to form additional antibodies with subsequent transfusions (5, 11-12), making it more difficult to provide compatible RBC for these patients. According to published Caucasian antigen frequencies (13) approximately 18% of ABO compatible blood donors would be suitable for this patient. This patient was consistently transfused with red cells which did not possess antigens for which she had antibodies, and K negative RBC whenever possible. On 9th July 2017 she was transfused a K positive unit which was crossmatch compatible. The K antigen is immunogenic (11) and the patient produced anti-K. She also produced Kell system antibodies to Kp^a and Js^a during her transfusion dependency and these were each presumably produced in response to a single exposure as these antigens are of low frequency in Caucasians (14). It was fortuitous that the patient possessed both the major antigens in the Duffy system and S and s in the MNS system (Table 2), thus rendering her unable to produce alloantibodies to these antigens.

More than a third of alloantibodies studied in large patient cohorts have been reported to disappear when antigenic stimulation is absent (10). This is normally associated with the Kidd blood group system (8), but in this patient was also seen with the Rh and Kell system alloantibodies. In 2008, Reverberi (10) reported that 50% of red cell alloantibodies could be predicted to survive for approximately 3 years and 7 months. In our case study the anti-E was detectable for 9 months (July 2013 to April 2014). The anti-K was detectable for 14 months (September 2017 to November 2018) and the anti-Jk^b was detectable for 3 months (July 2013 to October 2013). Alloantibodies produced by this patient disappeared much earlier than expected (10).

A positive DAT can be attributed to autoimmune haemolytic anaemia (15) or incompatible blood transfusion (16) and can also be caused by some medications (17). This patient had a positive DAT whenever tested (Table 3). She was on many medications that were prescribed both long-term and as needed, the latter including the antibiotic vancomycin. There have been cases of drug-induced haemolytic anaemia (DIHA) where antibodies to vancomycin have been identified (18,19). It is unknown whether this patient had DIHA as additional laboratory tests specific for this diagnosis were not carried out. It is also possible that the positive DAT results were due to incompatible red cells when blood group alloantibodies were at low levels early in their production. For example, from April 2014 to August 2016 (Table 1) an AUS was reported, meaning an antibody was present but was not giving a clear pattern of reactivity with the panel cells, making it impossible to identify an alloantibody or to provide red cells negative for the corresponding antigen. Later in June of 2017 anti-Kp^a was identified. Between April 2014 and August 2016 the patient received 36 units of RBC and it is possible that one of these was Kp^a positive and incompatible for the patient.

Although the positive DAT results may have been associated with HTR it is impossible to draw this conclusion because a pre-transfusion DAT could not be consistently performed as the patient almost always had transfused RBC in her circulation. The patient did not display any obvious symptoms of haemolysis and therefore there was no strong indication for the laboratory to investigate either DIHA or HTR. An eluate was performed on one occasion but showed no evidence of antibody specificity. A portion of patients and healthy blood donors present with a positive DAT without any explanation or risk factors and this is regarded as an insignificant finding in the absence of markers of haemolysis (17). This could be the case for this patient.

In conclusion, this case report presents a patient with complex co-morbidities who required regular transfusion of red blood cells. The appearance and the ephemeral nature of the red cell alloantibodies that complicated the management of this patient are familiar to transfusion scientists and clinicians. Fortunately, only a minority of patients (2% to 6%) requiring transfusion respond as strongly to red cell antigens as this patient did (2).

Table 1. Transfusion and antibody history of patient.

Time period	RCAS	Antibodies	Donor unit profile	# of units
December 2012	-	-	A, D +	3
Early July 2013	+	Anti-E	A, D+, E-	2
Late July 2013	+	Anti-E and anti-Jk^b	O, D + & D-, E -, Jk(b-)	4
October & November 2013	+	Anti-E and auto-antibody	No transfusion	-
April 2014	+	AUS	O, D +, E -, c -, K -, Jk(b-)	2
February 2015 – Early July 2016	-	-	A & O, D + & D -, E -, K -, Jk(b-)	27
Late July 2016 – August 2016	+	AUS	A & O, D + & D -, E -, K -, Jk(b-)	7
September 2016 – May 2017	-	-	A & O, D + & D -, E -, K -, Jk(b-)	37
June 2017 – August 2017	+	Anti-Kp^a	A & O, D + & D -, E -, K - & K+*, Jk(b-)	10
22 September 2017	+	Anti-K	A & O, D + & D -, E -, K -, Jk(b-)	2
25 September 2017 – January 2018	+	Anti-K & anti-Kp^a	A & O, D + & D -, E -, K -, Jk(b-)	14

1 February 2018	+	Anti-K	O, D +, E -, K -, Jk^b -	2
14 February 2018	+	Anti-K	No transfusion	-
Late February 2018 – Early March 2018	+	Anti-K & anti-Kp^a	A & O, D + & D -, E -, K -, Jk^b -	4
Late March 2018 – Early May 2018	+	Anti-Js^a, anti-Kp^a & anti-K	A, D + & D -, E -, K -, Jk^b -	3
Late May 2018 – 1 August 2018	+	Anti-K & anti-Kp^a	No transfusion	-
8 August 2018 – 29 August 2018	+	Anti-K	O, D -, E -, rr, K -, Jk^b -	2
September 2018 – 24 October 2018	+	Anti-K & anti-Kp^a	No transfusion	-
31 October 2018	+	Anti-Js^a, anti-K & anti-Kp^a	No transfusion	-
November 2018	+	Anti-K, anti-Kp^a & AUS	No transfusion	-
11 December 2018	+	Anti-Kp^a	O, D -, E -, rr, K -, Jk^b -	2
22 December 2018†	-	Anti-Js^a & anti-Kp^a	No transfusion	-
24 December 2018	+	Anti-Kp^a & AUS	O, D+, C+, c-, E-, e+, K-, Jk^b-	2
4-26 January 2019	-	-	A & O, D+ & D-, C+ & C-, c+, E-, e+, K-, Jk^b-	3
31st January 2019	+	Anti-Kp^a	A, D-, C-, c+, E-, e+, K-, Jk^b-	1
7 February 2019	+	AUS	O, D -, E -, rr, K -, Jk^b -	1

*One K+ unit transfused early July. AUS = antibody of undetermined specificity. RCAS = red cell antibody screen. After identification of anti-Kp^a and anti-Js^a, units were not phenotyped for these low frequency antigens, but were matched on the basis of indirect antiglobulin crossmatch compatibility with the units. † screen was negative in the presence of Anti-Js^a & anti-Kp^a as the screening cells did not express these antigens.

Table 2. Extended red cell phenotype of patient.

System	Antigens present
Rh	D, C, c, e
Duffy	Fy^a, Fy^b
Kidd	Jk^a
MNS	S,s

Table 3. DAT results of patient.

Date	Anti-IgG	Anti-C3
25th July 2013	1+	w+
26th September 2013	2+	w+
31st October 2013	3+	2+
10th April 2014	3+	2+
30th July 2016	1+	w+

DAT = Direct Antiglobulin Test. Scoring of reactions =0 to 4+ (3).

AUTHOR INFORMATION

Bhavisha Solanki, BMLS, Trainee Medical Laboratory Scientist[1]
Raewyn Cameron, DipMLT, Charge Scientist Transfusion[1]
Diane Matheson, DipMLT, Core Laboratory Scientist/2nd in Charge Transfusion[1]
William Perry, BSc FIBMS, Medical Laboratory Scientist[2]
Holly Perry, MSc MPhil PhD DipMLT, Senior Lecturer[3]

[1]PathLab, Rotorua
[2]Waitemata District Health Board, Auckland
[3]Auckland University of Technology, Auckland

Author for correspondence: Dr. Holly Perry.
Email: holly.perry@aut.ac.nz

REFERENCES

1. International Society of Blood Transfusion. Red cell immunogenetics and blood group terminology. 2019. Accessed 13th November, 2019 at http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology.
2. Hendrickson JE. Red blood cell alloimmunization: induction of immunity and potential mitigation strategies. *ISBT Science Series* 2018; 13: 105-111.
3. Fung MK, Grossman BJ, Hillyer CD, Westhoff CM. Technical Manual. 18th ed. American Association of Blood Banks, Bethesda, MD, 2014.
4. Liu C, Grossman BJ. Antibody of undetermined specificity: frequency, laboratory features, and natural history. *Transfusion* 2013; 53: 931-938.
5. Reid ME, Denomme GA. DNA-based methods in the immunohematology reference laboratory. *Transfus Apher Sci* 2011; 44: 65-72.
6. Hendrickson JE, Hillyer CD. Noninfectious serious hazards of transfusion. *Anesth Analg* 2009; 108: 759-769.
7. Sawada K, Fujishima N, Hirokawa M. Acquired pure red cell aplasia: updated review of treatment. *Br J Haematol* 2008; 142: 505-514.
8. Hamilton JR. Kidd blood group system: outwardly simple with hidden complexity. *ISBT Science Series* 2019; 14: 3-8.
9. Stack G, Tormey CA. Estimating the immunogenicity of blood group antigens: a modified calculation that corrects for transfusion exposures. *Br J Haematol* 2016; 175: 154-160.
10. Reverberi R. The persistence of red cell alloantibodies. *Blood Transfus* 2008; 6: 225-234.
11. Fluit CR, Kunst VA, Drenthe-Schonk AM. Incidence of red cell antibodies after multiple blood transfusion. *Transfusion* 1990; 30: 532-535.
12. Higgins JM, Sloan SR. Stochastic modeling of human RBC alloimmunization: evidence for a distinct population of immunologic responders. *Blood* 2018; 112: 2546-2553.
13. Harmening D. Modern Blood Banking and Transfusion Practices. 6th ed. Davis Company, Philadelphia, 2012.
14. Dean L. Chapter 8. The Kell blood group. In: Blood Groups and Red cell Antigens. www.ncbi.nlm.nih.gov/books/NBK2270/.

15. Packman CH. The clinical pictures of autoimmune hemolytic anemia. *Transfus Med Hemother* 2015; 42: 317-324.
16. Zantek ND, Koepsell SA, Tharp DR Jr, Cohn CS. The direct antiglobulin test: a critical step in the evaluation of hemolysis. *Am J Hematol* 2012; 87: 707-709.
17. Garratty G. The James Blundell Award Lecture 2007: do we really understand immune red cell destruction? *Transfus Med* 2008; 18: 321-334.
18. Gniadek TJ, Arndt PA, Leger RM, et al. Drug-induced immune hemolytic anemia associated with anti-vancomycin complicated by a paraben antibody. *Transfusion* 2018; 58: 181-188.
19. González CA, Guzmán L, Nocetti G. Drug-dependent antibiotics with immune hemolytic anemia in AIDS patients. *Immunohematology* 2003; 19: 10-15.

NZIMLS Membership

Member: Scientists registered with the Medical Sciences Council of NZ (includes 'Fellow' and 'Life' members)

Associate: Any person(s) not eligible for any other class of membership (includes Laboratory Technicians and non-vocationally qualified persons associated with the profession)

Student: Any person(s) currently enrolled in the third or fourth years of a BMLSc degree being taught at a NZ University

Membership of the NZIMLS provides the following benefits:

- A sense of professional belonging
- Preferential access to educational activities organised by the NZIMLS
- Discounted registration costs for attendance and/or participation in NZIMLS educational activities such as seminars, scientific meetings and workshops
- Opportunity to apply for The Barrie Edwards and Rod Kennedy Scholarship, and the NZIMLS Research Grant.
- Access to Fellowship, Medical Laboratory Technician and Specimen Services examinations
- All members receive issues of the NZ Journal of Medical Laboratory Science
- Opportunity to participate in the promotion and administration of the profession at the NZIMLS Council or SIG level
- Discounted costs for the NZIMLS CPD programme.
- Voting rights for elections to the Council of the NZIMLS (restrictions apply to some forms of membership).

NB: The NZIMLS Council considers all applications for membership at each of its quarterly meetings.

Application for membership is available at

https://www.nzimls.org.nz/shop

Membership FAQs

Do I have to be a member of NZIMLS?

Membership is voluntary. However, membership of your professional body is your first step toward your identity as both a health-care and a laboratory professional. Membership is open to those engaged in, training in, or associated with the profession of Medical Laboratory Science.

What type of membership do I apply for?

Scientists registered with the Medical Laboratory Sciences Council of NZ should apply for Full Membership with NZIMLS.
Technicians registered with the Medical Laboratory Sciences Council of NZ should apply for Associate Membership with NZIMLS.
Any person not eligible for Full Membership (e.g. non-registered Laboratory Technicians, trainees and non-vocationally qualified persons associated with the profession) may apply for Associate membership.

I'm a BMLSc student. Am I able to hold membership?

Yes! Student membership is available for anyone currently enrolled in their third or fourth year of a BMLSc degree being taught in a New Zealand university.

How can I apply for or renew membership?

You may apply for new membership or renew your current membership here.

Can I get reimbursed for my membership payment?

Some laboratories do reimburse their members who are also enrolled in our CPD programme. Check with your Supervisor if this applies to you.

Does membership with NZIMLS automatically register me with the Medical Sciences Council?

No. The Medical Sciences Council (MSC) and NZIMLS are two separate companies. To apply for registration, or to renew your Annual Practicing Certificate, please visit www.mscouncil.org.nz

I am emigrating to New Zealand. Does membership with NZIMLS help with employment?

No, membership with NZIMLS does not help if you are currently overseas looking to work in New Zealand.

CASE STUDY

Identification of *Legionella worsleiensis* in a five-year old male: a case report

Delphine Marjoshi and Aaron Keene

ABSTRACT

Legionellosis can present as a severe form of pneumonia which results from infectious *Legionella* species. Despite advances in the medical field, disease caused by *Legionella* still result in significant rates of mortality and morbidity worldwide. Each year a number of *Legionella* cases are reported in New Zealand, predominantly caused by *L. pneumophila* and *L. longbeachae*. Generally, it is thought that pneumonia caused by *Legionella* species is mainly among the adult demographic and only rarely in children. Here, we report a case of *L. worsleiensis* infection causing Legionnaires' disease in a five-year old male. The clinical presentation resembled infections caused by other species of *Legionella*. The diagnosis was made using PCR and the bacterial species was identified by 16S rRNA sequencing. Two other family members also presented with similar symptoms, however, the presence of *L. worsleiensis* could not be established. All three patients recovered after macrolide antibiotic treatment. To our knowledge, this is the first case of *L. worsleiensis* infection in a child in New Zealand. Furthermore, this case may be part of a point -source outbreak, which has never been reported for this species.

Key words: paediatric Legionellosis, *Legionella worsleiensis,* New Zealand, 16S rRNA sequencing.

N Z J Med Lab Sci 2020; 74: 144-147.

INTRODUCTION

Legionella are pathogenic Gram-negative bacteria that were first identified in 1977 to cause disease (1). These bacteria are found globally in aquatic and soil environments (2). In New Zealand, legionellosis was first diagnosed in 1979 (2). Compared to other countries, New Zealand has a high incidence of Legionnaires' disease (3) with approximately 150-200 or more cases per year reported in the last five years (4). There were 247 reported cases in 2016 and 221 reported cases in 2017 (4). The large majority of these cases are associated with two prevalent *Legionella* species, *L. longbeachae* and L. pneumophila (4).

According to the World Health Organization (WHO), 75% to 80% of reported Legionnaires' disease cases are found in patients who are 50 years or over (5). This trend is also observed in New Zealand (4), however, incidence rates among children are relatively unknown and it is therefore considered to be rare (3). In a study conducted by Dalton *et al.* (2018) in which 36 children presenting with lower respiratory tract infection in Christchurch over 16 months, only one patient was positive for *Legionella* (3).

Here, we present a case of community acquired legionellosis that was caused by an uncommon species of *Legionella, L. worsleiensis,* in a five-year old male in Christchurch, New Zealand. The mother and brother of the patient also presented with similar symptoms. *L. worsleiensis* was first isolated from water from a cooling tower in England (6). To our knowledge, there has only been one other reported case of *L. worsleiensis* infection, which was identified in an ICU patient in France (7). This species of *Legionella* has not been previously reported to cause disease in New Zealand.

CASE REPORT

A 35-year old woman, normally fit and well, presented to her GP in early December with a seven day history of fevers and productive cough. Her sputum sample was tested for *Legionella* on an in-house screening PCR (targeting a sequence common to all *Legionella* species), which showed the presence of *Legionella spp.* at a low level (crossing point value 39). A second pan-*Legionella* PCR confirmed the positive result but was negative for both *L. longbeachae* and *L. pneumophila* specific targets. Sequencing was not able to determine the *Legionella* species, presumably due to the low copy number of nucleic acid in the specimen.

One week later, her five-year-old son was taken to the same GP practice with a two-week history of fevers and a productive cough. The GP sent in a sputum sample requesting *Legionella* PCR with the accompanying clinical details of "maternal *Legionella*". The specimen was positive for *Legionella* DNA on the pan-*Legionella* screening PCR (crossing point value 35), and positive on the second pan-*Legionella* PCR (crossing point value 38), but was negative by species specific PCR for both *L. longbeachae* and *L. pneumophila*. 16sRNA gene primers specific for Legionella species (8) was used to generate a PCR product of 386 bp. The 386 bp sequence was compared with sequences in the GenBank database for identification using BLASTn method. The best match was a *L. worsleiensis* strain (GenBank number NR_044971.1), with a match of 99% (383 bp out of 386 bp). The next closest match was *L. quateirensis* (GenBank number NR_044965.1) with a match of 98% (378 bp out of 386 bp). A BLAST of the protein sequence gave *L. worsleiensis* as the best match. The programme Quick Bioinformatic Phylogeny of Prokaryotes leBIBI (https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi) was used to compare amino acids and a phylogenetic tree was produced (Figure 1). The 386 bp sequence was uploaded to GenBank with the following accession number: MT013285.

On further enquiry, the other child living in the same residence (seven-year old male) of the index case and her five-year old son also had a similar lower respiratory tract infection and fever, but was not able to produce a sputum sample. His GP requested urine antigen tests for *L. pneumophila* serogroup 1, *L. longbeachae* and *Streptococcus pneumoniae*, which were all negative. This test was done using the IMMUVIEW® *L. pneumophila* and *L. longbeachae* Urinary Antigen Test by SSI Diagnostica, which was under evaluation at the time and is no longer used at Canterbury Health Laboratories.

Public Health was notified of the single confirmed case of *L. worsleiensis* in the five-year old child, the confirmed low-level *Legionella* species in the mother and a clinically similar illness in the other sibling, all of whom shared the same residence. A health protection officer commenced an investigation which involved an examination of the water supply of the house and environmental sampling. Seven water samples were collected from two different water supplies (electric cylinder supplying the children's bathroom, and a gas water heater supplying the parents' bathroom), horse troughs, the home swimming pool and two decorative fountains at a separate residence. These samples were sent to ESR for *Legionella* culture (tested under ISO 11731 standard) but did not grow any *Legionella* species.

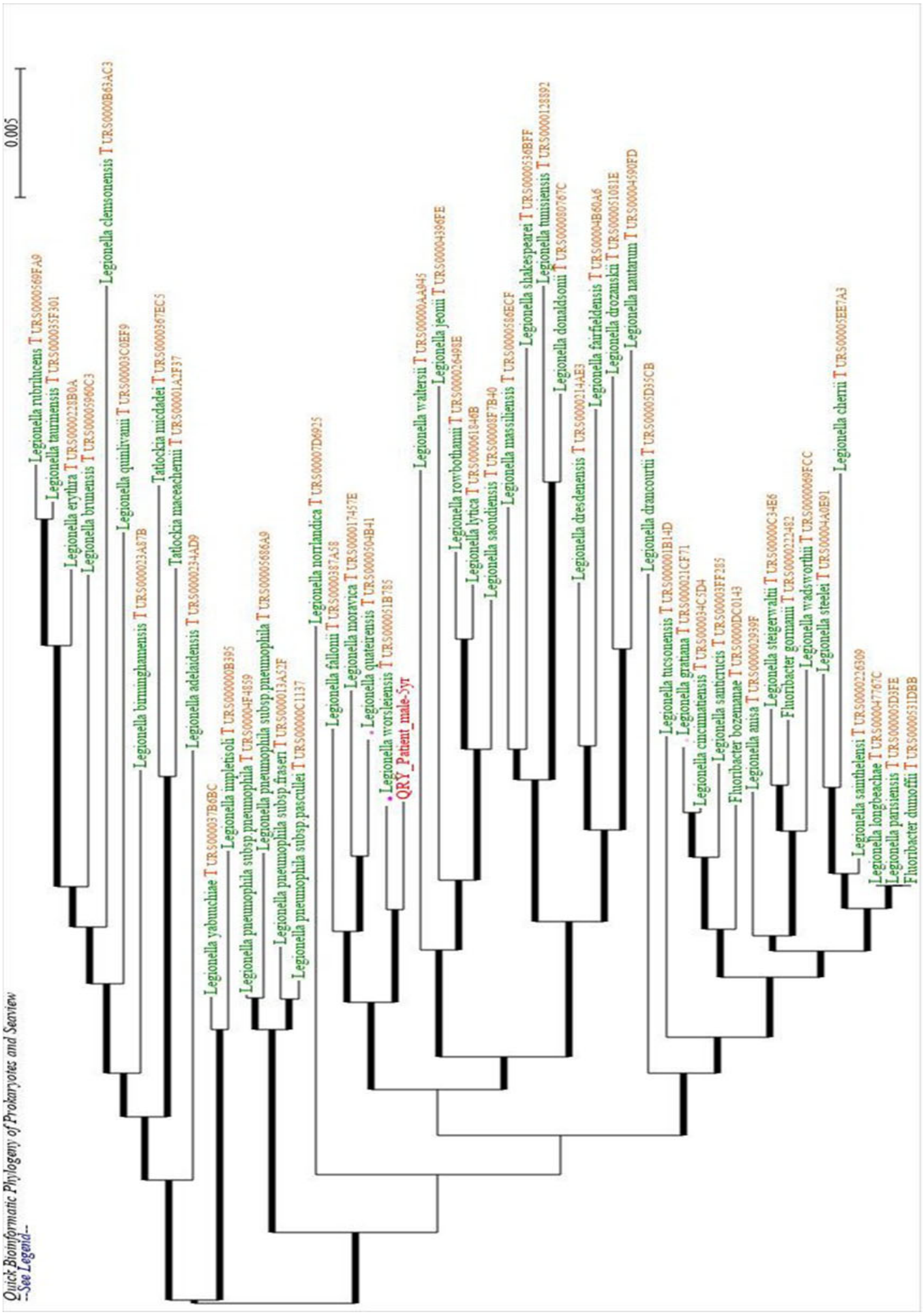


Figure 1. Phylogenetic tree of *L. worsleiensis* identified in the five-year old male (Red) based on 386bp sequence. The tree was constructed using the Quick Bioinformatic Phylogeny of Prokaryotes leBIBI programme

DISCUSSION

Since the discovery of *L. pneumophila* in 1977, more than 50 species of *Legionella* have been identified but only about half cause human disease (1). Various man-made environments act as reservoirs of *Legionella* and are often the source of human exposure (2). The general population is exposed to numerous pathogenic *Legionella* species in the environment; however, most cases are due to infection with *L. pneumophila, L. longbeachae* and *L. dumoffi* (2). Legionellosis can be either sporadic or associated with point source outbreaks. *L. pneumophila* outbreaks are associated with exposure to aquatic environments that harbour the bacteria, such as air conditioning units and whirlpool spas (9). *L. longbeachae* cases are associated with direct contact with soil, commercial potting mix or other decomposing material (10).

L. worsleiensis is known to be present in the aquatic environment. In a study in the Netherlands it was isolated from various surface and ground water (11). Non-*longbeachae*, non-*pneumophila* species have been associated with disease in humans, however, limited information is available on *L. worsleiensis* in the literature. To date, there is only one reported case of *L. worsleiensis* related human infection (7).

This patient was an adult and had a mixed infection with two other species of *Legionella*. The identification of *L. worsleiensis* in the five-year old child in this case study makes it the second reported case of *L. worsleiensis* infection in humans and the first in a child. The disease burden of *Legionella* is high in New Zealand; in particular, region of Canterbury has one of the highest rates (4). It mainly affects the adult population, surveillance data from 2017 in New Zealand showed that legionellosis in children is rare, 1.35% of cases were19-years old or younger (4).

Legionnaires' disease clinically presents as pneumonia (12). In adults there are a number of risk factors identified for Legionnaires' disease (12), which may help with a diagnosis. Risk factors include smoking, chronic obstructive pulmonary disease, diabetes, a compromised immune system and being the recipient of a transplant or chemotherapy (13). These risk factors are not normally applicable to the paediatric population and the most common symptoms are fever and cough, which are non-specific (14). This makes it harder for clinicians to suspect *Legionella* infection in the first instance. As reported by Greenberg *et al.,* a majority of children who develop Legionnaires' disease have an underlying disease or are immunosuppressed and are therefore likely to be tested for *Legionella* (14). The presence of *Legionella* in the respiratory tract is generally associated with disease, as opposed to colonisation (15). The detection of *L. worsleiensis* in the five-year old child in this case report suggests that *L. worsleiensis* can cause disease in healthy children. Increased awareness of *Legionella* species as a possible cause of paediatric pneumonia is warranted (14).

Despite one recent case with evidence for person-to-person transmission (16), *Legionella* is historically considered to spread from the environment, not by person-to-person transmission. The case in Canterbury was investigated as a possible point source outbreak given the onset of symptoms and the clustering within a single household. Although the mother was positive for *Legionella*, we were unableto establish if it was a *L. worsleiensis* infection. Two cases of legionellosis connected geographically and temporally are highly suggestive of a point source outbreak. The other seven-year child, who had a similar lower respiratory tract infection, could not be tested for *Legionella* genus or other species of *Legionella* as the patient could not produce a sputum sample. It is estimated that as many as half of the patients with lower respiratory tract infections do not produce sputum (15). As only a urine sample could be collected for this patient, Legionella infection could not be established. This highlights the limitation of urinary antigen tests, as many only test for *L. pneumophila* serogroup 1.

Despite efforts, the Public Health investigation could not ascertain the source of *L. worsleiensis* as samples were all *Legionella* culture negative. Many previous outbreak investigations were also unsuccessful in locating the source of *Legionella*, in Europe the source of *Legionella* could not be found in four of the ten largest *Legionella* outbreaks (17).There are a number of variables that can affect environmental testing of *Legionella*. These include slow growth rate, presence of interfering microbiota in the sample, loss of viability after sampling, day-to-day variability in the number of *Legionella* at a given source and transport conditions (18,19). *Legionella* also has the ability to convert to a viable but non-culturable state, making it harder to culture and can cause an underestimation of the number of *Legionella* present in a given sample (17,18). Laboratory expertise is also key in culturing *Legionella*; a survey conducted by the College of American Pathologists showed that one third of clinical laboratories were unable to grow a pure *Legionella* culture (19). Despite the challenges in culturing *Legionella*, outbreak investigations are crucial in preventing further spread of the disease. These investigations also increase our understanding of *Legionella* ecology and epidemiology (19).

CONCLUSIONS

We report a case of *L. worsleiensis* infection in a five-year old male in New Zealand, an unspeciated *Legionella* infection in his mother and a compatible illness in his brother, tested for *L. pneumophila* serogroup 1 and *L. longbeachae* only. To our knowledge this is the second report of *L. worsleiensis* infection but the first reported infection in a child. It also raises the possibility of *L. worsleiensis* causing point source outbreaks. *Legionella* infections in children are considered to be rare and are often under- reported, this case report highlights the need for increased awareness of *Legionella* as a possible cause of paediatric pneumonia. This case also emphasises the importance of speciation for Public Health and epidemiological surveillance. We hope that this case report will increase awareness among clinicians and the scientific community about *L. worsleiensis*.

ACKNOWLEDGMENTS

We sincerely thank Trevor Anderson, Scientific Officer - Canterbury Health Laboratories, who conducted 16S rRNA sequencing and analysed the sequence data. We also thank the Virology and Microbiology staff at Canterbury Health Laboratories, for performing the tests. We wish to thank Julie Creighton, Senior Scientist and Clinical Lecturer, University of Otago, Department of Microbiology, Canterbury Health Laboratories, for reading and editing the manuscript. We would also like to thank ESR for providing valuable surveillance data for New Zealand.

AUTHOR INFORMATION

Delphine Marjoshi, MSc, Medical Laboratory Scientist
Aaron Keene, BMLS MBChB, Microbiology Registrar

Canterbury Health Laboratories, Christchurch, New Zealand

Correspondence: Delphine Marjoshi.
Email: Delphine.Marjoshi@cdhb.health.nz

REFERENCES

1. Amodeo MR, Murdoch DR, Pithie AD. Legionnaires' disease caused by Legionella longbeachae and Legionella pneumophila: comparison of clinical features, host-related risk factors, and outcomes. *Clin Microbiol Infect* 2010; 16 (9): 1405-1407.
2. Graham FF, White PS, Harte DJ, Kingham SP. Changing epidemiological trends of legionellosis in New Zealand, 1979-2009. *Epidemiol Infect 2012*; 140(8): 1481-1496.
3. Dalton STP, Wen SCH, Murdoch DR, Martin JF, Wallace KN, Vivian CA, et al. Searching for pediatric Legionnaire disease in a population with high adult incidence. *Pediatr Infect Dis J* 2018; 37(9): 960-961.
4. The Institute of Environmental Science and Research Ltd. Notifiable Diseases in New Zealand: Annual Report 2017. 2019.

5. WHO. Legionellosis. https://www.who.int/news-room/fact-sheets/detail/legionellosis2018.
6. Dennis PJ, Brenner DJ, Thacker WL, Wait R, Vesey G, Steigerwalt AG, et al. Five new Legionella species isolated from water. *Int J Syst Bacteriol* 1993; 43(2): 329-337.
7. Berger P, Papazian L, Drancourt M, La Scola B, Auffray J-P, Raoult D. Ameba-associated microorganisms and diagnosis of nosocomial pneumonia. *Emerg Infect Dis* 2006; 12(2): 248-255.
8. Reischl U, Linde HJ, Lehn N, Landt O, Barratt K, Wellinghausen N. Direct detection and differentiation of Legionella spp. and Legionella pneumophila in clinical specimens by dual-color real- time PCR and melting curve analysis. *J Clin Microbiol* 2002; 40(10): 3814-3817.
9. Fields BS, Benson RF, Besser RE. Legionella and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev* 2002; 15(3): 506-526.
10. Whiley H, Bentham R. Legionella longbeachae and legionellosis *Emerg Infect Dis* 2011; 17(4): 579-583.
11. Wullings BA, van der Kooij D. Occurrence and genetic diversity of uncultured Legionella spp. in drinking water treated at temperatures below 15 C. *Appl Environ Microbiol* 2006; 72(1) :157-166.
12. Yu VL, Lee TC. Neonatal legionellosis: the tip of the iceberg for pediatric hospital-acquired pneumonia? *Pediatr Infect Dis J* 2010; 29(3): 282-284.
13. Farnham A, Alleyne L, Cimini D, Balter S. Legionnaires' disease incidence and risk factors, New York, New York, USA, 2002–2011. *Emerg Infect Dis* 2014; 20(11): 1795-1802.
14. Greenberg D, Chiou CC, Famigilleti R, Lee TC, Yu VL. Problem pathogens: paediatric legionellosis—implications for improved diagnosis. *Lancet Infect Dis* 2006; 6(8): 529-535.
15. Murdoch DR. Molecular genetic methods in the diagnosis of lower respiratory tract infections. *APMIS* 2004; 112(11-12): 713-727.
16. Borges V, Nunes A, Sampaio DA, Vieira L, Machado J, Simões MJ, et al. Legionella pneumophila strain associated with the first evidence of person-to-person transmission of Legionnaires' disease: a unique mosaic genetic backbone. *Sci Rep* 2016 ;6: 26261.
17. Cebrián F, Montero JC, Fernández PJ. New approach to environmental investigation of an explosive legionnaires´ disease outbreak in Spain: early identification of potential risk sources by rapid Legionella spp immunosensing technique. *BMC Infect Dis* 2018; 18(1): 696.
18. Díaz-Flores Á, Montero JC, Castro FJ, Alejandres EM, Bayón C, Solis I, et al. Comparing methods of determining Legionella spp. in complex water matrices. *BMC Microbiol* 2015; 15(1): 91.
19. Mercante JW, Winchell JM. Current and emerging Legionella diagnostics for laboratory and outbreak investigations. *Clin Microbiol Rev* 2015; 28(1): 95-133.

APRIL 2019 JOURNAL QUESTIONNAIRE ANSWERS

1. Limitations of prostate specific antigen testing has resulted in what?

   Large numbers of unnecessary biopsies and overtreatment.

2. What are the possible mechanisms of antimicrobial resistance cases?

   Multiple, including reduced permeability to antibiotics, increased efflux pumps, changes in antibiotic targets by mutation, or modification of target enzymes.

3. Which types of glycopeptide resistance genes have been described in *Enterococci* and which are the most important?

   vanA, vanB, vanC, vanD, vanE and vanG. vanA and vanB are the most important.

4. How can vancomycin resistance genes be transmitted and potentially cause what?

   Transmitted from enterococcus to staphylococcus through plasmids. Potentially causing serious life-threatening infections.

5. What is the principle of the cyanmethemoglobin photometric method for determining haemoglobin?

   Haemoglobin is converted to cyanmethemoglobin after the addition of potassium cyanide and ferricyanide, absorbance is then measured at 540nm by a spectrophotometer against a standard solution.

6. What are currently the most important diagnostic criteria for appendicitis?

   Rely on clinical manifestations and laboratory tests, such as white cell count and their differentiation.

7. Norovirus is a virus with a RNA genome that encodes open reading frames. What do those frames encode?

   Three open reading frames: ORF1 encodes the replicate polyprotein, ORF2 encodes the major capsid proteins, ORF3 encodes the minor capsid proteins.

8. Maternal deficiencies of Vitamin B12 and folate during pregnancy have been implicated in which fetal developmental complications?

   Neural tube defects and megaloblastic anaemia.

9. What are the New Zealand recommendations for dietary supplementation of Vitamin B12 and folate in pregnancy?

   For folate: 0.8mg daily for at least four weeks before and twelve weeks after conception, and the consumption of folate rich foods. For Vit B12: 2.6mcg to 2.8 mcg daily.

10. The metabolic consequences of refeeding syndrome are characterised by what processes in the body?

    Deranged phosphate, potassium, magnesium, and fluid balance within the body.

Correction to November 2019 Questionnaire question 7 answer:

7. Which factors have been identified as important modifiable metabolic syndrome risk factors?
   Many factors, including physical inactivity, obesity, excessive alcohol consumption and unhealthy diet

Abstracts from Semester 2, 4th year Otago BMLSc student research projects

Optimisation of Alpha-1 Antitrypsin genotyping assay at Wellington SCL

Kendra Caddy[1] and Chor Ee Tan[2]
[1]University of Otago, Dunedin and [2]Wellington Southern Community Laboratories, Wellington

Objectives: To optimise the Alpha-1 Antitrypsin assay used at Wellington SCL. This involved optimising the singleplex PiZ assay on the LightCycler 2.0 and creating a multiplex assay that can be used on the LightCycler 480 analyser.
Methods*:* Initially, changes were made to the Capillary LightCycler 2.0 singleplex PiZ assay to improve the melting peaks. These changes included different concentrations of primers to favour the reverse primer, along with different concentrations of probes, favouring the mutation specific probe. The reaction was multiplexed with the PiS assay and alterations were made to the multiplex mastermix to optimise amplification of both targets. This was done by favouring the PiS reaction by increasing the PiS primer concentration. The PiS reaction required an advantageous change because the PiZ reaction has preferential amplification due to the smaller product size. Finally, the mastermix was tested with various genotypes. This included MM, MS, MZ, SZ and ZZ genotypes. There were no SS genotypes available for testing.
Results: The peaks were greatly improved in the singleplex PiZ reaction with clear distinction seen between heterozygote and homozygote variants along with wildtype genotypes. The multiplex reaction was created and produced readable peaks for each of the genotypes tested.
Conclusions: The project was successful in that a new test procedure was created. The singleplex reaction is being used for diagnostic testing in Wellington SCL in favour of the previous singleplex test. Ideally; in the future, modifications should be made on the multiplex reaction to make the genotypes more distinguishable.

The clinical performance of the Panther Fusion *Bordetella pertussis* assay

Otto Dove[1], Judy Moodie[2] and Gayleen Parslow[2]
[1]University of Otago, Dunedin, [2]Southern Community Laboratories, Dunedin

Objectives: Despite the implementation of a funded vaccination schedule, whooping cough remains a significant health issue in New Zealand. Recently, Hologic has released a *Bordetella pertussis* assay for the Panther Fusion system, bringing the benefits of random-access to whooping cough analysis. The aim of this study was to determine the sensitivity and specificity of the Hologic Panther Fusion's *B. pertussis* assay, compared to the Meridian Bioscience illumigene and Roche Diagnostic LightCycler assays. The secondary aim of this study was to determine the sensitivity and specificity of a variety of transportation media, to determine if they can be implemented onto the Panther Fusion system.
Methods: A mixture of 93 inpatient and outpatient samples were obtained for analysis. These samples were analysed by either the Meridian Bioscience illumigene or Roche Diagnostics LightCycler. A sample of the specimen (450uL) was transferred to a Hologic specimen lysis tube. The lysis tube was then inserted into the Hologic Panther Fusion, which detected the presence of *B. pertussis* through a real-time polymerase chain reaction. The target of analysis was the IS481 region, found in high levels in the *B. pertussis* genome.
Results: The Panther Fusion performed with an overall specificity of 100% (94.4%-100%) and sensitivity of 96% (80.5%-99.3%). The specificity of the universal transportation medium (UTM), dry swabs, and charcoal swabs was 100%. The UTM and dry swabs were equal for assay sensitivity. Due to a limited number of samples, the sensitivity of the charcoal swab method was unable to be determined.
Conclusion: The Panther Fusion's *B. pertussis* assay performed excellently when compared to the Meridian Bioscience illumigene and Roche Diagnostics LightCycler. This allowed Southern Community Laboratories Dunedin to perform *B. pertussis* requests on their Panther Fusion, bringing the benefits of random-access to whooping cough analysis.

Determining the predictive power of the immature platelet fraction parameter in identifying patients with a *JAK2* V617F mutation in myeloproliferative neoplasms

Jeshyloria Flores[1,2], Kevin Taylor[2] and Peter Edwards[2]
[1]University of Otago, Dunedin and [2]Canterbury Health Laboratories, Christchurch

Objectives: The recent revision of myeloproliferative neoplasms (MPNs) category in the 2016 World Health Organization (WHO) classification system has directed alterations to the diagnostic criteria in polycythaemia (rubra) vera (PV). The recent lowering of the haemoglobin (Hb) and haematocrit (Hct) thresholds has led to a markedly multiplied proportion of haematologists review referrals. Consequently, some laboratories have utilised algorithms using immature platelet fraction (IPF) and other complete blood count (CBC) parameters to efficiently screen patients for PV. This study investigated the predictive power of IPF in identifying patients with *JAK2* V617F mutation in myeloproliferative neoplasm as way to efficiently select cases for haematologist review.
Methods: A total of 92 samples (n = 59) with MPNs were included in the study. IPF assessment was performed on 47 *JAK2*-positive patients, 22 *JAK2*-negative patients, and the remaining 23 who did not have *JAK2* mutation analysis done. The obtained reference interval for IPF on the Sysmex XN-2000 was obtained from 44 healthy adults.
Results: The 95% reference interval for IPF on the Sysmex XN -2000 was 1.8–12.7×10^9/L (90% confidence intervals (CIs) were 0.6–3.0 and 11.5–13.9, respectively). Both *JAK2*-positive and *JAK2*-negative patient groups who were not on hydroxyurea treatment showed similar IPF levels. There was no significant statistical difference in IPF levels between groups (p = 0.473).
Conclusion: The IPF analysis paired with CBC algorithmic approach did not satisfy the proposed predictive power of IPF in identifying *JAK2*-positive MPNs. Employing the algorithmic approach in *JAK2* mutation referrals may not be the cost-effective way in identifying patients with suspected *JAK2*-related MPNs.

Establishing a reference interval for the immature platelet fraction parameter: a posteriori study

Sarah Grant[1] and Richard Parker[2]
[1]University of Otago and [2]Southern Community Laboratories, Dunedin

Objectives: The immature platelet fraction (IPF) is a parameter not routinely reported, but one which can provide an indirect index of the rate of thrombocyte production in the marrow, analogous to reticulocytes in erythropoiesis. The aim of this project was to establish a reference interval for the immature platelet fraction parameter and assess the potential usefulness in identifying abnormal thrombopoietic disorders.

Methods: A retrospective review of 3,668 specimens from June and July 2019 were obtained from the Sysmex XN 3000 analyser. Exclusion criteria were applied and prospective samples assessed by the project supervisor for inclusion. Further investigation of potential outliers was assessed and removed from the selection. A total of 121 specimens were selected and statistically analysed using both parametric and non-parametric methods. Additionally, patients with clinical indications of immune thrombocytopenia were also collated to compare against the proposed reference intervals.
Results: The patient data comprised of 72 (59.5%) females and 49 (40.5%) males with an age ranging from 16 to 93 years. The reference interval for IPF produced by parametric methods was 1.3 – 10.7%, whereas non-parametric methods produced a reference interval of 1.1 – 9.5%. The IPF values obtained from patients with immune thrombocytopenia were statistically distinct from those of the reference cohort, with a positive predictive value (PPV) of 0.80 and a false detection rate (FDR) of 0.20 using parametric methods, compared to a PPV of 0.83 and a FDR of 0.27 using non-parametric methods
Conclusion: Both reference intervals were comparable in terms of clinical utility, due to overlapping confidence intervals and similar pathological disorder detection; however, non-parametric methods performed better overall. Reassessment with a larger dataset from a healthy cohort or a prospective study would be beneficial in increasing the reliability of the reference interval.

Comparison of information delivery methods for informed consent for blood transfusions

April Jones[1] and Krishna Badami[2]
[1]University of Otago, Dunedin and [2]New Zealand Blood Service, Christchurch

Objectives: Informed consent is a process in which patients are educated about their treatment options, allowing them to make autonomous decisions about whether they consent to treatment. Blood transfusions are a treatment option associated with a multitude of risks which require patient consent. There is evidence the information provided for informed consent, particularly around the risks associated with blood transfusions, is not adequately understood by patients. This project aimed to investigate whether the process of patient education can be improved by use of an information sheet.
Methods: A randomised controlled trial was performed using members of the public and medical staff. Participants were randomly assigned to the control or intervention group. The control group received an audio recording replicating the current education process. The intervention group received an A4 sheet of information. Understanding and recall of the information provided was assessed using a questionnaire. The mode for each group was calculated and used to compare the survey results.
Results: The results implied neither form of information was adequate in promoting understanding of the risks associated with blood transfusions. There was some evidence suggesting the intervention improved understanding and recall of the frequency of transfusion associated risks. The severity of such events appeared to be unclear irrespective of the type of information received.
Conclusion: With further development of the information given, routine use of supplementary paper based information could assist understanding of the frequency and severity of transfusion associated risks through reinforcement of information given during a discussion.

The effect of refrigeration on sample integrity for prothrombin time coagulation assays

Sophia Jordan[1] and Rhonda Lucas[2]
[1]University of Otago, Dunedin and [2]Southern Community Laboratories, Dunedin

Objectives: The Clinical Laboratory Standards Institute guidelines advise against testing prothrombin Ttime on samples refrigerated for 4 hours or longer due to the activation of FVII when samples are stored between 2-8 degrees Celsius. The aim of this project was to assess the validity of these claims and to determine if the duration of refrigeration significantly affected results for a number of samples.
Methods: The Sysmex CS-2500 automated coagulation analyser was used to test prothrombin time, and the reagent used was Siemens Dade Innovin. Patient samples were collected in sodium citrate tubes and centrifuged to form plasma on packed red cells. Routine prothrombin time values for each patient were noted and re-tested on the same machine after 2 hours, 4 hours and 8 hours refrigeration time. As there was a limited amount of useable volume per sample, a separate set of control samples were left for the same periods of time at room temperature.
Results: Results were displayed as the total percentage drift from the initial prothrombin time value, which was clinically significant if the mean % drift was >10%. The drift did increase in proportion to refrigeration time but was below the >10% cut off for all time periods. This is consistent with other similar studies, one of which even said there was no >10% change following up to 24 hours of refrigeration time. If future studies were continued, it would be interesting to assess the effect of even longer periods of refrigeration on prothrombin time, and to determine if the change was clinically significant.
Conclusion: Refrigeration time up to 8 hours at 4 degrees Celsius did not change prothrombin time values to a clinically significant degree. This implies that, when required, it is acceptable for this laboratory to run additional prothrombin time tests on samples which have had up to 8 hours of refrigeration time.

An evaluation of the solid phase red cell adherence method for elution studies

Rafaella Liew[1], Mark Howard[2] and Jared Pratt[2]
[1]University of Otago, Dunedin and [2]Pathlab, Tauranga

Objectives: Elution studies in transfusion science are commonly performed following a positive direct antiglobulin test result. While the use of Solid Phase Red Cell Adherence (SPRCA) technology has been established to be effective in detecting antibodies in serum samples, few studies have investigated the comparability of the SPRCA and microcolumn methods for testing of eluates. The use of SPRCA would offer practical advantages if adopted in a blood bank setting. The objective of our study was to investigate whether SPRCA technology is comparable to microcolumn methods for the detection of antibodies eluted from red cells.
Methods: In our study, 13 samples which underwent elution testing by manual ORTHO Biovue manual microcolumn methods were identified on blood bank computer software and tested using SPRCA elution screen on the Immucor NEO Iris. The period of the study was two months. Results were analysed using concordance analysis.
Results: Of 13 samples there were 12 concordant cases (92%), 8 concordant positive (62%) and 4 concordant negative (31%). There was one discordant case which was positive by SPRCA technology only. This sample had an antibody of undetermined specificity identified by standard antibody screen and panel results on the serum sample.

Conclusion: The two methods were comparable according to this study, a conclusion supported by the few other studies examining the comparability of the two methods. The small sample size of our study limited the statistical interpretation. Our conclusions are only applicable to the two conditions which affected samples in our study, namely autoantibodies and presence of passive maternal antibody in newborns. Based on our results, SPRCA technology is comparable to microcolumn technology for the detection of antibodies in eluates.

Carbapenemase detection method comparison: mCIM vs Rapidec Carba NP vs Mast Carba Pace

Xavier Lozada[1], Jacquie Leaman[2], Catherine Gordon[2] and Yin-Tzu Wang[2]
[1] University of Otago, Dunedin and [2]Canterbury Southern Community Laboratories, Christchurch

Objectives: The aim of this study was to compare three different methods that detect carbapenemase produced by Enterobacterales, *Pseudomonas* species and *Acinetobacter* species. The three methods were the modified Carbapenem Inactivation Method (mCIM), RAPIDEC CARBA NP and Mast CARBA PAcE. This comparison was done in terms of their sensitivity, specificity, advantages and disadvantages.
Methods: Thirty-eight isolates were collected from Canterbury SCL (CSCL), Canterbury Health Laboratories (CHL) and Wellington SCL (WSCL). Twenty-nine of these isolates were confirmed to be carbapenemase producers by the Institute of Environmental Science and Research (ESR). The nine negative isolates were collected from CSCL. In batches of five, test organisms were cultured on a Columbia sheep blood agar plates and incubated for 18-24 hours at 35 degrees Celsius. On the second day, the mCIM test was performed and a purity plate of the test organism prepared using Columbia sheep blood agar. Plates were incubated overnight. On the third day, mCIM results were recorded and the RAPIDEC CARBA NP and the CARBA PAcE tests carried out using the fresh isolates from the purity plates. The methods for each kit were followed according to the manufacturers' package insert.
Results: The mCIM had a sensitivity of 93.1% and a specificity of 100.0. The CARBA NP had a sensitivity of 82.8% and a specificity of 100.0. The CARBA PAcE had a sensitivity of 93.1% and a specificity of 100.0%.
Conclusion: The CARBA NP and CARBA PAcE kits were rapid, but significantly more expensive than the mCIM. The mCIM method was very cheap, however, it had a slow turnaround time. The mCIM method and CARBA NP were highly accurate for detecting carbapenemase producing Enterobacterales but both tests had difficulty detecting OXA-type carbapenemase in *Acinetobacter baumannii*. The CARBA PAcE test was highly accurate for detecting Enterobacterales and *Acinetobacter* species, but it was unable to detect PAM- or POM-type carbapenemase in *Pseudomonas* species.

Instrument comparison between 2014 and 2018 models of the HemoCue® 501 HbA1c point-of-care analyser

Constantine Mao[1] and Heather Hughes[2]
[1]University of Otago, Dunedin and [2]Canterbury Southern Community Laboratories, Christchurch

Objectives: Certain haemoglobin variants interfere with HbA1c results obtained from ion-exchange high performance chromatography. An alternative assay method was required to measure the HbA1c levels in these cases. At Canterbury SCL, a HemoCue 501 HbA1c point-of-care analyser was used as the alternate test method. However, the 2014 HemoCue model used at Canterbury SCL required patient samples to be run in duplicate due to its poor precision. HemoCue released a newer 2018 model that claimed to have better precision than previous models. The aim of this study was to compare the precision between the 2014 and the 2018 HemoCue 501 analyser models.
Methods: Twenty K_2EDTA whole blood patient samples were collected and tested over a period of three weeks. Each sample was tested three times in total, once on the 2014 model and twice on the 2018 model. The coefficient of variation (CV) was calculated for the 2018 model using the twenty patient duplicate results and six QC duplicate results. The CV for the 2014 model was calculated using 227 duplicate results from previous patients' records.
Results: The mean CV of the 2018 model was 2.7% (*n*=27). The mean CV of the 2014 model was 9.1% (*n*=227). The mean difference between the 2018 and 2014 results was 2.45.
Conclusion: The newer 2018 HemoCue had better precision with a CV of 2.7% compared to the 9.1% CV of the older 2014 model. However, the new model had results on average 2.5 mmol/mol higher than the older model. Further testing is recommended to investigate the higher results.

Method validation for a total bile acids assay

Jia Xin Mok[1], Jenny Chu[2], Maxine Reed[2], Philippa Holdaway[2] and Joanne Webb[2]
[1]University of Otago, Dunedin and [2]Wellington Southern Community Laboratories, Wellington

Objectives : Total bile acids (TBA) have emerged as a significant biomarkers to evaluate liver function, hepatobiliary diseases and obstetric cholestasis. Serum TBA level measurement can therefore be a useful test to be introduced into clinical biochemistry laboratories. The purpose of this study was to validate the use of a new TBA in serum assay on the Roche-Hitachi Cobas c501 at Wellington Southern Community Laboratories (WSCL)
Methods: Within-day precision (percentage coefficient of variation (%CV) and average deviation) were examined using two patient serum samples, one of a lower and another of a higher value. Accuracy was analysed by obtaining correlation with the external QC (EQC) values given by RANDOX and average deviation between the values. Correlation of the TBA assay (RANDOX Laboratories Ltd.) was determined by comparing the TBA results of the patient serum samples (n=13) from Canterbury Health Laboratory (CHL) with the TBA results obtained at WSCL.
Results: The TBA assay displayed a precision of 2.49%CV for sample 1 and 1.26%CV for sample 2 and average deviation of 0.106 µmol/L and 0.479 µmol/L respectively. A correlation coefficient, R^2 value of 0.9997, was obtained with an average deviation of 2.605µmol/L between the EQC target values and EQC results obtained. An R^2 value of 0.9804 was determined for the correlation between the TBA results from CHL and WSCL.
Conclusion: This TBA assay displayed excellent precision of %CV less than 10% and small average deviation values for both the samples. Excellent correlation of 0.9997 R^2 value obtained shows accuracy of results with the EQC target values and R^2 value of 0.9804 determines excellent correlation between both the CHL and WSCL results. However, limitations of this experiment such as insufficient patient samples should to be taken into consideration.

An investigation of fibrinogen assay stability at Hutt Southern Community Laboratories

Roseanna Olifent[1] and Brice Thomson[2]
[1]University of Otago, Dunedin and [2]Wellington Southern Community Laboratories, Lower Hutt.

Objectives: The fibrinogen assay at Hutt SCL has demonstrated unexplained quality control (QC) failure. The aim of this study was to investigate causes and remedies, removing the need for 4-hourly changes of Dade Owren's Veronal Buffer (OVB). It was investigated whether the in-use period of abnormal fibrinogen QC vial could be extended from 2 to 5 days.

Methods: Reagents and QC Citrol 1 and abnormal fibrinogen were prepared as per manufacturer protocols. QC was run on a CS2100i coagulation analyser every 2-4 hours, for up to 24 hours, while one vial of OVB remained onboard. Certain variables were modified: OVB refrigerated between QC runs; OVB closed versus open vials between QC runs; and reagent changes following QC failure. A precision check was performed. One vial of abnormal fibrinogen QC was run daily for 10 days.
Results: All 5 runs demonstrated acceptable Abnormal fibrinogen QC results (range: 0.98-1.27g/L; allowable limits: 0.85-1.25g/L) and a systematic negative drift in Citrol 1 results (range: 5.24 -11.02% drop until QC failure, between 4h, 42m and 9h 57m). The precision check passed (CV = 2.3%). Citrol 1 or thrombin replacement did not correct the drift (average decreases of 0.28g/L and 0.059g/L respectively) while fresh OVB did (positive shift of 0.37g/L, 0.77g/L and 2.6/L; allowable limits: 2.25-2.73g/L). Refrigerating OVB, Citrol 1 had an insignificant positive drift over 14 hours (0.05g/L). Closed vial OVB caused a positive (0.9g/L) and open vial a negative drift (0.04g/L). Abnormal fibrinogen results were acceptable across 10 days: 0.96-1.19g/L.
Conclusion: High ambient temperature is a likely cause of OVB becoming rapidly unstable, resulting in QC failure. Evaporation may also contribute. Further investigation is recommended. OVB on-board stability time should not be extended beyond 4 hours. Abnormal fibrinogen QC vial in-use time can be extended to 5 days.

An evaluation of the accuracy and precision of the Gonotec Osmomat 3000

Isla Revell[1], Hank Ploeg[2], Elly Barnes[2], Chris Leaver[2], Berlin Mariadhas[2] and Jenny Castle[2]
[1]University of Otago, Dunedin and [2]Canterbury Health Laboratories, Christchurch

Objectives: The Advanced 3250 Osmometer used at Canterbury Health Laboratories provides high quality osmolality results. However, this method is time consuming, uses a large sample volume and requires manual entry of results into Delphic. The Gonotec Osmomat 3000 is a modern, affordable, user friendly instrument that can produce timely results, uses a smaller sample volume and eliminates manual data entry by communicating directly to Delphic, achieving a paperless laboratory. The aim of this investigation was to evaluate the performance of the Gonotec Osmomat 3000 compared to the Advanced 3250 Osmometer. Should the new analyser produce results of similar or better quality than the current, the Gonotec Osmomat 3000 will replace the Advanced 3250 Osmometer at Canterbury Health Laboratories.
Methods: Twenty urine samples and twenty plasma/serum samples were collected and tested on both analysers; these tests were performed in duplicate and compared to evaluate accuracy and precision. Quality control checks were performed every four hours on both analysers.
Results: The average coefficient of variation for the Advanced 3250 Osmometer was 0.3% (1.3 mOsm/kg variation between duplicate samples) with an accuracy of 0.8% as opposed to the Gonotec Osmomat 3000 which had an average coefficient of variation of 1.7% (8.2 mOsm/kg variation between duplicate samples) and an accuracy of 4.7%. The Advanced 3250 Osmometer therefore produced more reliable results than the Gonotec Osmomat 3000 according to the laboratory analytical performance specifications.
Conclusion: Based on the findings from this investigation, the Gonotec Osmomat 3000 is not an appropriate replacement for the Advanced 3250 Osmometer at Canterbury Health Laboratories.

Evaluation of the Thermo Scientific CEDIA cyclosporine PLUS low range reagent

Kahla Tyson[1] and Christian Christian[2]
[1]University of Otago, Dunedin and [2] Southern Community Laboratories, Dunedin

Objectives: Cyclosporine is an immunosuppressant which works by impairing T lymphocyte activation. It is mainly used post transplantation to minimise risk of rejection. Cyclosporine has no therapeutic range but needs monitoring as many factors can affect its blood concentration and bioavailability. The assay is a homogenous enzyme immunoassay which measures between 25 and 450 ng/ml. The aim of this investigation was to assess this specific cyclosporine assay using inter-precision, intra-precision, linearity and RCPA samples.
Methods: Within run precision was tested by repeat testing the high and low QC samples ten times. The between run precision was performed by testing the 3 QC levels supplied by the company a total of 21 times over 5 weeks. The coefficient of variation was calculated for both sets of results. Linearity was evaluated using a two-fold serial dilution of the high QC level and RCPA samples were tested and compared to the RCPA report received for SCL Dunedin.
Results: The coefficients of variation (CVs) for intra-precision were 4.3% and 2.4%, and for inter-precision they were 7.1%, 4.8% and 3.0% respectively for each QC level of increasing concentration. These were all under 10% indicating this assay is precise. The dilution of QC3 showed good linearity with an r value of 0.9997. The RCPA samples showed variable accuracy to desired results.
Conclusion: From the data collected, it was shown that the Thermo Scientific CEDIA cyclosporine PLUS assay low range reagent is a good assay in terms of precision and linearity and could be used within a laboratory to detect cyclosporin concentrations in patient samples.

Investigating national cancer cases 2014 to 2019, with analysis of expected cancer cases for Wellington SCL

Kaitlyn Ustin[1] and Khoi Phan[2]
[1]University of Otago, Dunedin and [2]Wellington Southern Community Laboratories, Wellington

Objectives: The main focus of this investigation is on the greater Wellington region, with a specific goal of estimating cancer cases expected in the next five years at Wellington laboratory to allow staffing and equipment preparations to be made.
Methods: When a new cancer is identified, the pathologist assigned reports the case to the Ministry of Health, which manages a database of all cancer registration details stored in spreadsheet form. Examination of this data allowed for the identification of trends to be found. These trends can help identify future rates of cancer cases by projecting the common trends throughout the years and combining this with demographic data also available from the government.
Results: Historically, cancer cases in Wellington have increased by 8.63% each year, so it can be assumed cases will continue to increase by roughly 8.63% each year going forward. The aging of the population as well as the population growth rate must also be considered. The most prominent age group for cancer registration is individuals aged 64 years ±10 years. The ethnic group with the highest total cancer registrations are NZ Europeans; however, Maori have the highest normalised rate of cancer.
Conclusion: In the future, the cancer registration rates are expected to increase by at least 8.63% per year over the next five years. The whole of New Zealand should be preparing for an increase in the workload needing to be processed within the next five years.

A methylation specific high-resolution melt assay for the detection of mosaicism in Fragile X Syndrome patients

Rachel van Dyk[1] and Tony Raizis[2]
[1]University of Otago, Dunedin and [2]Canterbury Health Laboratories, Christchurch

Objectives: To determine the sensitivity of an in-house methylation specific high-resolution melt assay for the detection of methylation mosaicism present in individuals with Fragile X Syndrome.
Methods: DNA extracted from EDTA blood samples underwent an in-house bisulfite treatment. A mixing study was conducted using a normal allele male with an unmethylated *FMR1* promoter and full mutation allele male with a methylated *FMR1* promoter. Mixtures of the male samples were made in 10% increments and were then tested using an in-house methylation specific high-resolution melt assay. Controls used were a female with two normal alleles and a female with one full mutation alleles and a normal allele. The two female samples were also mixed at a 1:1 ratio. All samples and mixtures were tested in duplicate.
Results: Comparing the expected and observed level of fluorescence, the assay was shown to be reliable down to 10%. A linear regression of the midpoint of the melt curves at 71.1^{0}C showed a strong correlation with an R squared value of 0.98. The male mixtures produced pronounced double sigmoidal curves while the female mixture in comparison produced a barely discernible double sigmoidal curve.
Conclusion: The high level of correlation between the fluorescence of expected and observed for the melt curves suggests that the assay will be suitable to detect methylation differences caused by size and/or methylation mosaicism. Differences in the male and female melt curve shape is likely caused by differences in the methylation density of the *FMR1* promoter due to differences in the methylation mechanisms due to the full mutation expansion of the CGG repeat and of X-inactivation.

Comparison of agar and plasma cell blocking methods for cytological investigation

Aysha Willis[1,2], Paul Spek[2], Esther Ooi[2], and Rhiannon Brown[2]
[1]University of Otago, Dunedin and [2]Southern Community Laboratories, Dunedin

Objectives: To compare cell block methods for cytological investigation of fluid specimens using nutrient agar and plasma-thrombin methods. Specimens (16) were prepared in parallel for comparison.
Methods: Each specimen was poured into a 50mL tube and centrifuged to form a cell pellet. The supernatant was discarded. Plasma-thrombin method samples were then washed in PBS with 1mL of 0.02% toluene blue and centrifuged to reform cell pellets. Supernatants were discarded. Plasma-thrombin samples had four drops of 100 units thrombin and two drops of normal pooled plasma added. Specimens were centrifuged for 1 minute. Plasma-thrombin samples were given time to coagulate then were dislodged with the addition of 10% formalin. Clots were coaxed onto surgipath surgical paper and wrapped before being placed into a histology cassette. The agar method samples had the supernatant discarded after the first centrifugation, cells were loosened from the pellet by vortex mixing. Four drops of molten nutrient agar were added. Samples were set before being coaxed onto a histology cassette. Specimens were embedded into paraffin wax and stained with an H & E method. Slides were examined using a search grid pattern. Cells were counted at 40 x magnification for 10 fields of view. Cell counts were compared statistically using a single tailed t-test.
Results: Sixteen plasma- thrombin and the 16 agar specimens were compared for cellularity. Results were analysed using a single tailed t-test. Results showed a significant difference (P= 0.005531) in cellularity between the two methods.
Conclusion: The plasma-thrombin method has a significantly greater cell yield from each of the specimens collected and should be considered the procedure for cell blocking at the Dunedin Cytology Laboratory.

Optimisation of a Dako 22C3 PD-L1 antibody protocol on the Ventana Benchmark ULTRA in the diagnosis of non-small-cell lung cancer

Hannah Wong[1] and Spencer Walker[2]
[1] University of Otago, Dunedin and [2]Southern Community Laboratories, Dunedin

Objectives: The aim of the study was to develop and optimise a protocol on the Ventana Benchmark ULTRA analyser using the gold standard Dako 22C3 PD-L1 antibody clone for the detection of PD-L1 in non-small-cell lung cancer (NSCLC).
Methods: Seven protocols were tested. Each protocol's slide had sections of NSCLC patient tumour tissues previously graded to have tumour proportion scores (TPS) of 70%, 5%, and 1%. Tonsil and/or cell line controls were used on each slide. The variables tested were Cell Conditioning 1 solution incubation time, primary antibody dilution and primary antibody incubation time. The TPS of slides were determined and the staining quality graded against NordiQC guidelines by pathologists.
Results: The average TPS of all protocols for the Ventana TPS 70% section were all above 50%, with the protocol with the closest TPS to the target 70% being U30 at 72.5%. The Ventana TPS 5% and Ventana TPS 1% sections were given TPS <1% by all protocols. Meanwhile, protocol U30 gained the highest staining quality scores, with two pathologists grading it as optimal and one as good. All other protocols were graded as good or worse.
Conclusion: Of the protocols developed, the U30 protocol was the most ideal protocol. However, further testing in the form of technical repeats, testing sections with different levels of PD-L1 expression, as well as further changes in the variables are required in order to develop a more optimal protocol for the use of the Dako 22C3 PD-L1 antibody to be used on the Ventana Benchmark ULTRA.

Establishing Ret-He cut-offs for diagnosis of functional iron deficiency

Charlotte Duen-Yi Wu[1] and Robert Allan[2]
[1]University of Otago, Dunedin and [2]Canterbury Southern Community Laboratories, Christchurch

Objectives: Functional iron deficiency (FID) occurs when iron cannot be efficiently incorporated into erythroid precursors. It is a condition commonly present in infectious diseases, malignant diseases, and plays an important role in anaemia of chronic disease. Currently laboratories lack a parameter that can accurately diagnose FID; however, many researchers have suggested using reticulocyte haemoglobin equivalent (Ret-He). Our primary aim was to determine a Ret-He cut off for diagnosis of FID, and the secondary aim was to determine a Ret-He cut-off that could differentiate between classical and FID.
Methods: Samples were collected from patients with chronic kidney disease and anaemia. Serum ferritin, haemoglobin and Ret-He were measured to classify samples into FID, classical iron deficiency (CID) and normal. Ret-He results were analysed using t-test, receiver operating characteristics (ROC) curve.
Results: The difference between the sample mean Ret-He in FID and normal individuals was significant. Similarly, a significant difference was found between the sample mean

Ret-He of FID and CID patients. Ret-He cut off_of <30.8 pg was obtained to identify FID ($p<0.0001$). However, it was not possible to obtain a clinically significant cut off to identify patients who have CID as opposed to FID. **Conclusion:** A potential Ret-He cut off for FID was obtained, but to exclude patients with absolute iron deficiency further investigations are required.

Preliminary method validation for AFP, CEA, CA125 and hCG in pleural and peritoneal fluids on the Roche Cobas 8000 e602 unit

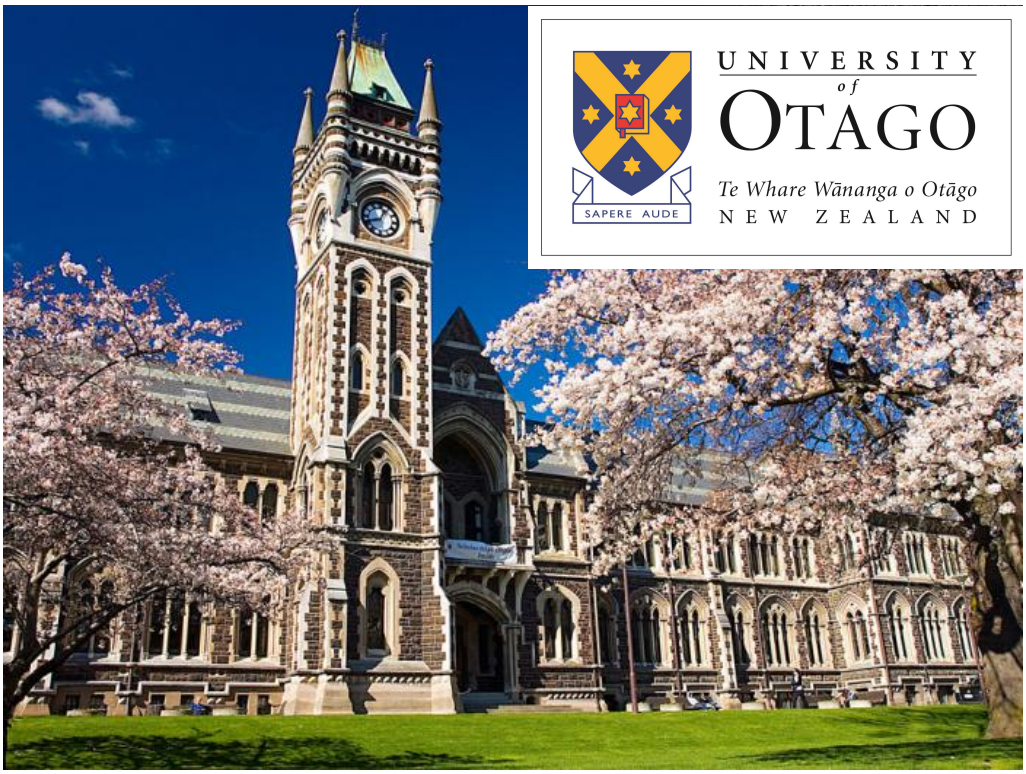
Annabella C Yee[1], Sian Horan[2] and Jason Wong[2]
[1]University of Otago, Dunedin and [2]Southern Community Laboratories, Dunedin

Objectives: This study was to investigate the suitability of using serum AFP, CA125, CEA and hCG+β assay for the analysis of pleural and ascitic fluid samples using the Roche Cobas e602 immunoassay unit based on an electrochemiluminescent sandwich immunoassay principle. **Methods:** Between-batch precision and stability were measured on analytes 10 times over seven days from pooled pleural and ascitic fluids. For within-batch precision, analytes were measured 10 times in one run. To measure recovery and linearity, samples were spiked with 10% high concentrate analyte. Expected values were calculated from serum and fluid tumour marker concentration before and after spiking of analyte. For linearity, samples were diluted on-board 2-fold, 5-fold, 10-fold and 20-fold with saline. Acceptable limits were based on RCPA allowable limits of performance. Results obtained were compared to the manufacturer's method sheet for serum assays. **Results:** Both within-batch and between-batch precision, and recovery were within 10% for all tumour markers AFP, CA125, CEA and hCG. Stability over 7 days at 4°c was within acceptable limits. Linearity was acceptable for up to upper limits measured. **Conclusion:** Serum AFP, CA125, CEA and hCG were suitable assays for pleural and ascitic fluid. However, a higher AFP range and a larger range of fluids must be further investigated to address all components of fluid validation.

Masson's trichrome methods comparison

Jeff Zhong[1] and Kevin Shin[2]
[1]University of Otago, Dunedin and [2]Pathlab, Tauranga

Objectives: Masson's trichrome is the most common special staining method used in histology laboratories for the differentiation of collagen fibres from a target tissue, such as kidney and skin. Three different molecular size dyes are used to stain nucleus, muscle fibre, collagen fibre and erythrocyte. The aim of this methods comparison study was to identify whether the crystal Ponceau dye is preferred by the pathologist over acid fusion dye, as well as to identify the relationship between the staining intensity and the increased acid dye staining duration.
Methods: Skin, kidney and colon specimens were used in this study to demonstrate smooth muscle, collagen fibre and erythrocyte. Each test slide contained one section of colon, kidney and skin. In total, 24 slides were prepared grouped into eight sets. Four sets were used for the crystal Ponceau stain with four different staining durations and the other four sets were used for acid fusion stain. All methods were repeated three times to ensure reproducibility. Slides (24) were randomly labelled with an alphabetic letter from A to X to ensure a blind study. These slides were viewed by the in-house registrars, pathologists and senior scientists, and the top five preferred slides were voted after they had viewed all the 24 slides.
Results: Increasing staining duration showed no obvious trend with staining intensity for both acid dyes methods and the crystal Ponceau was the preferred dye of the viewers.
Conclusion: This study showed that 62% of the participants preferred crystal Ponceau stained slides, and no clear evidence showing staining duration has a direct relationship with stain intensity. Therefore, crystal Ponceau should not be replaced by acid fusion for Masson's trichrome protocol.



Science Digest

Contributed by Michael Legge

Long term immune impacts of measles infections in unvaccinated children

Prior to vaccination measles accounted for millions of deaths world-wide, primarily in children. Despite vaccination programmes measles is still affecting >7 million people with >100,000 deaths annually. Recently measles has made a significant resurgence primarily due to a lack of vaccination. Previous research has shown that following measles infections there is compromised immunity and depletion of memory T, B and plasma cells, which has been described as "immune amnesia". Recent collaborative research involving groups from Europe and the USA has identified that in those following measles infections any pre-existing immunity to other pathogens is diminished (1). In the current research the researchers collected bloods from families in the Netherlands that had low vaccination rates during a measles outbreak. A total of 77 unimmunized children were sampled prior to and after 10 weeks following infection. Of these 34 identified with mild measles and 43 with severe measles. Epitope specific repertoires were analyzed using VirScan, which detects antibodies to short contiguous epitopes. The researchers created a VirScan library, which encoded the full proteomes of approximately 400 species and strains of known human pathogenic viruses, plus many bacterial proteins. In addition they derived an epitope-binding signal, which provides a relative measure of antibody titre for each epitope. The findings indicated that measles infections created large reductions of both the antibody repertoire and magnitude of the binding signal. The authors commented that the measles virus could infect 20 to 70% of memory cells, including B, T, and plasma cells within 3 to 10 days after the initial infection. Commenting on historic measles infections the authors note that since the 1940s measles has the potential for immune-suppression and development of auto –immune related disorders. The potential time period for re-building the immune system following measles infections was considered to likely be years, which would place individuals at increased risk for other infections due to "immunity amnesia".

How 'catty-mice' help to solve the sexual cycle of *Toxoplasmosis gondii*

Toxoplasmosis gondii infections occur in approximately one-third of the human population. It also affects the developing fetus leading to blindness, intellectual handicap and hydrocephaly. The asexual cycle of *T. gondii* can occur in any mammal if contaminated water or food is consumed, forming cysts in the muscle and the brain. The sexual cycle is restricted to the cat intestinal epithelium ultimately producing environmentally resistant oocysts. The molecular basis for the specificity for the cat was, until recently, not known. During the infection in the cat the parasite undergoes a series of complex transformations resulting in oocysts that are stable to many environmental conditions and chemical disinfectants. Research from the USA has provided substantial evidence for the host specificity and development of the *T. gondii* sexual cycle in the cat (2). Using cat intestinal organoids researchers simulated *T. gondii* infections. Initially the results were not favourable and it was considered that a nutritional factor was missing. Subsequently oleic and linoleic acids were identified as the missing nutritional factors allowing merozoite development, which subsequently developed to oocysts. As cats are the only mammals to lack the enzyme, delta-6-desaturase, in the small intestine (a rate limiting enzyme to convert linoleic acid to arachidonic acid) it was considered this might be the biochemical factor for *T. gondii* development. This was supported, in part, with analysis of cat serum, which was 25 to 46% linoleic acid of the total fatty acid concentration, significantly higher than rodents and humans. To test the hypothesis that linoleic acid is the factor for hosting the development of *T. gondii* the authors inhibited delta-6-desaturase in mouse intestine tissue culture and subsequently in live mice (nick-named 'catty mice'), which resulted in the sexual stages of *T. gondii* developing and the mice remained infective for up to three months. The authors concluded that host diet is an important interaction for parasitic infections and that understanding theseinteractions would lead to the development of therapeutics.

Auto-brewery syndrome (ABS).

A 46 year old man with no significant psychiatric or medical history complained of memory loss, mental health changes and depression for over six years following treatment with cephalexin for a traumatic thumb injury while working for a construction company restoring hurricane damaged houses. Initial changes after treatment were reputed to be depression, 'brain fog' and development of aggression. He was referred to a psychiatrist who medicated him. Whilst under the care of the psychiatrist he was arrested for presumed driving while intoxicated. His initial blood alcohol level was 200mg/dl and although he denied using alcohol, the police refused to believe him. He recovered in hospital and was discharged. He then sought independent medical advice where further investigations were undertaken. While his bloods were normal his stool sample cultured *Saccharomyces cervisiae* and *S. boulordi* which were sensitive to azoles and nystatin. The patient was given a carbohydrate meal and after 8-hours his blood alcohol was 57mg/dl. He was treated initially with fluconazole, which was ineffective, and changed to nystatin where he improved. Subsequently he had episodic flare-ups, one of which resulted in a fall which required transfer to a neurosurgical unit for an inter-cranial bleed. During this time his blood alcohol levels were 50 to 400mg/dl. The medical staff refused to believe he was not drinking alcohol. After discharge his symptoms became worse and he sought help from the authors of the present case report (3) who collected gastrointestinal secretions by endoscopy. Fungal cultures of *Candida albicans* and *C. paarapsilosis* were obtained from the upper small intestine and caecal secretions. Initial treatment with itraconcazole was unsuccessful and was changed to iv micafungin, which was successful. He was started on probiotics, which were gradually increased in the range of bacteria, but no fungi were included in the pro-biotics. The patient is now asymptomatic, eating a normal diet and regularly checks his breath alcohol levels. The authors considered that the initial fungal infections were most likely mould contamination from the damp houses and concluded that individuals reporting similar symptoms and deny alcohol consumption should be investigated for ABS.

Note: The New Zealand upper limit blood alcohol level for driving is 50mg/dl.

REFERENCES

1. Mina MJ, Kula T, Leng Y, Li M, de Vries RD, Knip M, et al. Measles virus infections diminishes preexisting antibodies that offer protection from other pathogens. *Science* 2019; 366: 599-606.
2. Martorelli Di Genova B, Wilson SK, Dubey P, Knoll LJ. Intestinal delta-6-desaturase activity determines host range for Toxoplasma sexual reproduction. *PLoS Biol* 2019; 17(8): e3000364.
3. Malik F, Wickremesinghe P, Saverimuttu J. Case report and literature review of auto-brewery syndrome: probably an undiagnosed medical condition. *BMJ Open Gastroenterol* 2019; 6(1) :e 000325.

THE Pacific WAY

Centre based training courses

Due to COVID-19 and the closure of Pacific borders including that of New Zealand, the Pacific Pathology Training Centre (PPTC) unfortunately has had to transfer all Pacific students to its 2021 course calendar and also place on hold all Pacific in- country visits that were scheduled for 2020, until borders are re-opened. Centre based courses and their training dates for 2021 will be as follows:

- Laboratory Health and Safety and Quality Management Systems
  1 March – 26 March 2021 (4 weeks)
- Biochemistry
  19 April – 14 May 2021 (4 weeks)
- Effective Laboratory Management
  24 May – 18 June 2021 (4 weeks)
- Haematology and Blood Cell Morphology
  28 June – 6 August 2021 (6 weeks)
- Microbiology
  23 August – 18 September 2021 (4 weeks)
- Blood Transfusion Science
  18 October – 12 November 2021 (4 weeks)

For further information contact:
Navin Karan, Programme Manager
PO Box 7013, Wellington, New Zealand
Phone: +64 4 389 6294.
Email: navink@pptc.org.nz. www.pptc.org.nz

What measles and COVID-19 exposed in the Pacific!

Every nation around the globe has struggled and been shaken by the infective threatening force of COVID-19. The Pacific Island Nations were no exception. In the South Pacific, Samoa was still reeling from the effects of a devastating measles epidemic when the pandemic started to emerge worldwide. It had already activated outbreak control measures in an effort to prevent a second virus impacting the country. Many Pacific Island Countries and Territories were bracing themselves to be potentially overwhelmed by mass infection rates particularly so in the sample collection centres and laboratory testing sites.

Suddenly the calibre and status of national diagnostic laboratory services for all the Pacific nations was in the spotlight, looking exposed, and highly vulnerable. The need for skilled professionals, rapid response teams, essential testing facilities and kits, collection processes, computer data records, rapid testing analysers and efficient quality approved processes has dramatically raised the profile of medical laboratories to the forefront of healthcare services and a frontline essential tool needed in controlling all public health threats.

In the height of this crisis the PPTC was asked to respond on behalf of a NZAid Emergency Funding grant. We were called upon because our working relationship and technical expertise was already well established in Samoa and well acquainted with the laboratory's service capabilities. Also, of vital importance was the associated network of New Zealand diagnostic companies that the PPTC could source items from.

An urgent request had come from the laboratory management in Apia to upgrade and supply a wide scope of essential laboratory equipment to both the Upolu and Savaii sites. Eventually, over an intense four week period, equipment was installed into the laboratory sites which included four blood gas units, one Sysmbex 1000 haematology analyser, a BD FX blood culture machine, a BD Phoenix microbiology system, a centrifuge unit for the blood transfusion section, an XN550 coagulation instrument and three C311 biochemistry analysers. Although this equipment was not specifically for the testing of measles or coronavirus, it has proved invaluable in supplying essential tests associated with secondary infection and non-communicable diseases.

A huge effort to make this happen was directed and orchestrated by Navin Karan and PPTC staff with exceptional assistance given by BD, Roche, Fort Richard Laboratories, Radiometer NZ, and the wonderful Samoan laboratory staff.

The Tokelau's

The vulnerability of small Pacific Nations to infectious diseases, such as the measles outbreak experienced in Samoa or indeed COVID 19 as a global pandemic which continues to destroy human life in its path, has prompted requests of support from the New Zealand Government by Pacific nations to assist in their preparedness against such devastating events.

One nation in particular is Tokelau, a self-governing territory of New Zealand comprising of three atolls - Atafu, Nukunonu and Fakaofo and is situated approximately 500 km north of Apia, Samoa, the nearest port. The New Zealand Government has responded to a request of support for the establishment of a diagnostic laboratory in the Tokelau's that offers a greater comprehensive test menu, inclusive of COVID19 testing through Gene Expert Technology. This laboratory will also have general diagnostic capacity to meet community needs.

The PPTC has been asked by New Zealand's Ministry of Foreign Affairs and Trade to facilitate the procurement of a laboratory that is fit for purpose designed with appropriate internal structure, instrumentation, and consumables.

Can you help?

If any New Zealand medical laboratories have items of diagnostic instrumentation that have been recently upgraded or continue to be stored in the laboratory but are actually surplus to requirements, the PPTC would be most grateful if such items could be donated through its Centre to Pacific Island laboratories where there is an exceptional need. Pacific laboratories have very restricted budgets and often cannot afford to replace troublesome instrumentation that continues to breakdown and which is often discontinued because it is so outdated.

The PPTC would also welcome teaching resources in terms of wall charts, haematology case studies (stained blood films), projector slides, textbooks and journals (within 10 yrs of publication) etc , for teaching purposes in the Pacific, if you no longer have a use for them. Any contribution is so valuable to us. Please contact:

Phil Wakem
Chief Executive Officer
Pacific Pathology Training Centre
Wellington, New Zealand
E-mail: pptc @ pptc.org.nz or phil @ pptc.org.nz.
Tel: 64-4-389 6294 or 027 2305483.

Publications by NZIMLS members

This column is to showcase recent peer-reviewed publications by NZIMLS members. If you have had a recent publication please provide full details to the Editor at rob.siebers@otago.ac.nz.

Jones E, Burrell P, Barnett T, Lyons-Ewing D, Nuttall E. Erysipelothrix rhusiopathiae bacteraemia in an immunocompromised host: the unexpected complication of a crustacean altercation. *New Zealand Medical Journal* 2019; 12: 1504.

Vesty A, McAuliffe G, Roberts S, Henderson G, Basu I. Mycoplasma genitalium antimicrobial resistance in community and sexual health clinic patients, Auckland, New Zealand. *Emerging Infectious Diseases* 2020; 26: 332-335.

McAuliffe GN, Bissessor L, Basu I, Smith S, Upton A. Barriers to testing and management of Mycoplasma genitalium infections in primary care. *International Journal of STD and AIDS* 2019; 30: 1116-1123.

Irinyi L, Hu Y, Hoang MTV, Pasic L, Halliday C, Javawardena M, Basu L, et al. Long-read sequencing based clinical metagenomics for the detection and confirmation of Pneumocystis jirovecii directly from clinical specimens: A paradigm shift in mycological diagnostics. *Medical Mycology* 2019; pii: myz109.

Brown P, RELISH Consortium (including Siebers R), Zhou Y. Large expert-curated database for benchmarking document similarity detection in biomedical literature search. *Database* 2019; 2019: baz085.

Fox-Lewis A, Isteed K, Austin P, Thompson-Faiva H, Wolfgang J, Ussher JE. A case of imported Q fever in New Zealand. *New Zealand Medical Journal* 2019; 132(1505): 92-94.

Brewer N, Bartholomew K, Maxwell A, Grant J, Wihongi H, Bromhead C, et al. Comparison of two invitation-based methods for human papillomavirus (HPV) self-sampling with usual care among un- and under-screened Māori, Pacific and Asian women: study protocol for a randomised controlled community trial to examine the effect of self-sampling on participation in cervical-cancer screening. *BMC Cancer* 2019; 19(1): 1198.

Pullon BM. An evaluation of glycated haemoglobin elutions in zone 10 on capillary zone electrophoresis. *Journal of Laboratory Medicine* 2020; 44: 55-58.

Howard JC, Creighton J, Ikram R, Werno AM. Comparison of the performance of three variations of the Carbapenem Inactivation Method (CIM, modified CIM [mCIM] and in-house method (iCIM)) for the detection of carbapenemase-producing Enterobacterales and non-fermenters. *Journal of Global Antimicrobial Resistance* 2020; 21: 78-82.

Camburn AE, Petrasich M, Ruskova A, Chan G. Myeloblasts in normal bone marrows expressing leukaemia-associated immunophenotypes. *Pathology* 2019; 51: 502-506.

Chien N, Petrasich M, Chan G, Theakston E, Ruskova A, Eaddy N, et al. Early treatment of acute promyelocytic leukaemia is accurately guided by the PML protein localisation pattern: real-life experience from a tertiary New Zealand centre. *Pathology* 2019; 51: 412-420.

2020 NZIMLS CALENDAR
Dates may be subject to change

DATE	COUNCIL	CONTACT
19-20 August	Council Meeting, Rangiora	fran@nzimls.org.nz
DATE	**SEMINARS**	**CONTACT**
Under the present global circumstances, we are unsure if Special Interest Group meetings will proceed. Please keep an eye on our website for updates.		
NZIMLS ANNUAL GENERAL MEETING		
20 August, Christchurch 1pm. Members will be emailed further details.		
DATE	**MEMBERSHIP INFORMATION**	**CONTACT**
January	Membership and CPD enrolment due for renewal	sharon@nzimls.org.nz
31 January	CPD points for 2020 to be entered before 31 January	cpd@nzimls.org.nz
15 February	Material for the April issue of the Journal must be with the Editor	rob.s bers@otago.ac.nz
15 June	Material for the August Journal must be with the Editor	rob.siebers@otago.ac.nz
18 June	Nomination forms for election of Officers and Remits to be with the Membership (60 days prior to AGM)	fran@nzimls.org.nz
8 July	Nominations close for election of officers (40 days prior to AGM)	fran@nzimls.org.nz
26 July	Ballot papers to be with the membership (21 days prior to AGM)	fran@nzimls.org.nz
01 August	Annual Reports and Balance Sheet to be with the membership (14 days prior to AGM)	sharon@nzimls.org.nz
09 August	Ballot papers and proxies to be with the Executive Officer (7 days prior to AGM)	fran@nzimls.org.nz
15 September	Material for the November Journal must be with the Editor	rob.siebers@otago.ac.nz
DATE	**NZIMLS EXAMINATIONS**	**CONTACT**
07 November	QMLT Examinations	fran@nzimls.org.nz

Journal Questionnaire

Due to reduced opportunities to obtain CPD points due to the COVID-19 situation there are two journal questionnaires for the August 2020 issue. You can either do one (A) or the other (B) questionnaire, or both (A & B). There are 5 CPD points per questionnaire, thus if you do both and get at least 8 out of 10 questions right for each questionnaire then you get 10 CPD points. Otherwise, 5 CPD points for either questionnaire A or B.

Read the articles carefully as most questions require more than one answer. Answers are to be submitted through the NZIMLS website. Make sure you supply your correct email address and membership number. It is recommended that you write your answers in a word document and then cut and paste your answers on the web site.

You are reminded that to claim valid CPD points for successfully completing the journal questionnaire you must submit an individual entry. It must not be part of a consultative or group process. In addition, members who have successfully completed the journal questionnaire cannot then claim additional CPD points for reading the articles from which the questions were derived.

The site will remain open until Friday 2nd October 2020. You must get a minimum of eight questions right per questionnaire to obtain five CPD points. The Editor sets the questions but the CPD Co-Ordinator, Jillian Broadbent, marks the answers. Direct any queries to her at cpd@nzimls.org.nz .

AUGUST 2020 JOURNAL QUESTIONNAIRE (A)

1. Phenotypic tests for detection of carbapenemase production are prone to false negative results due to what?
2. The CARBA PAcE test is based on what test principle?
3. Fox P3 has a role in controlling what, acts as a negative regulator of what, and what does it suppress?
4. What are Tregs necessary for?
5. Why is the hyperinsulinaemic-euglycemic clamp test not useful in clinical application and epidemiological investigation?
6. What are thought to be the benefits of increased levels of haemoglobin F in sickle cell anaemia?
7. What defines the clinical features of sickle cell anaemia?
8. Where is the site of bleeding due to subdural haemorrhage, and what is it usually the result of?
9. What is enolase, how does it act, and what is it composed of?
10. Which aspects of work practice can be considered in everyday ethical concepts?

AUGUST 2020 JOURNAL QUESTIONNAIRE (B)

1. Why is detection of the nuclear dense fine speckled pattern for DFS70 antibody important?
2. Sensitivity of microscopy for the diagnosis of fungal infections varies with what?
3. What would help monitoring extraction efficiency of fungal DNA in clinical samples?
4. Which *Legionella* species are in the majority associated with Legionnaires' disease in New Zealand?
5. What are the risk factors for Legionnaires' disease in adults?
6. In antibody screening patients' plasma is tested against red cells that express which major red cell antigens?
7. What does alloimmunization to red cell antigens depend on?
8. What can a positive direct antiglobulin test be attributed to?
9. Platelet activation in diabetes increases the risk of what?
10. What is thought to give rise to platelet activation, what does this promote, and how is this reflected?

Note: The questionnaires have been set up separately online.

Questionnaire A may be found at
https://www.nzimls.org.nz/august-journal-questionnaire-a

Questionnaire B may be found at
https://www.nzimls.org.nz/august-journal-questionnaire-b

English to Māori Clinical Terms

Terms Relating to Clinical Conditions	
English	**Māori**
Anaemia	Mate rino
Appendicitis	Mate weu, whēkau kaka
Arteriosclerosis	Pakeke oteia totowhero
Artery	Iaia mai i te Manawa
Arthritis	Mate uruumu pona, mate kaikōiwi
Autopsy	Tirotiro tūpāpaku
Bacteria	Moroiti, kitakita, huakita
Blood bank	Pūtea toto
Blood clot	Katinga toto, kaitoto
Blood group	Tūmomo toto
Blood pressure	Rere ote iatoto, taukapa ote toto
Blood transfusion	Whāngai toto
Cancer	Mate pukupuku
Cancer cervical	Mate pukupuku ote waha taiawa
Cancer therapy	Whakatā mate pukupuku
Cardiac arrest	Manawatū, manawahē
Chicken pox	Koroputu hei
Coronary	Mate Manawa
Cot death	Mate pouraka
Cramp – legs	Kaurapa
Diabetes	Mate huka
Diabetic	Matehuka
Disease	Mate, tahumaero
Gene	Ira
German measles	Kōpukupuku
Glaucoma	Papahewa
Gonorrhoea	Mate paipai
Gout	Koute, porohau, puhipuhi
Haemophilia	Mate toto tepekore
Haemorrhage	Ikura, tahe toto
Heart attack	Manawatū, mate, Manawa, ma-nawahē
Hepatitis	Mate kowhai, mate ate kakā
Hypertension	Takawhita
Infection	Mate
Infertility	Pākoko
Leukemia	Mate ruru toto
Malignant	Kino, whakamate
Melanoma	Kiritona pukupuku, tonapuku
Meningitis	Kiriuhi ua kaka
Measles	Karawaka, mītera
Mumps	Mate kōpuku korokoro, mate pupu-hirepe
Pneumonia	Niumōnia, pūkaku kaka
Prostate	Repe tātea, repe ure
Respiratory disease	Mate roma hā
Rheumatic fever	Pīwa rūmātiki
Rheumatism	Rūmātiki. Kaikōiwi, uhumona
Rheumatoid arthritis	Kaikōiwi
Schizophrenia	Pōauau, wairua tuakoi
Schizophrenic	Wairua tuakoi
Syphilis	Pākewakewa
Tuberculosis	Matekohi-ā-kiko, kohitu
Urinary tract infection	Mate roma mimi

Terms in the Clinic	
English	**Māori**
Accident	Aituā
Asthma	Huangō, kume, hiki, tīmohu, ngio
Baby	Pōtiki, pēpi pēpe
Bandage	Tākai
Born/family	Whānau
Cancer	Mate pukupuku
Cough	Maremare
Blood/Bleed	Toto
Die/illness/infection	Mate
Doctor	Tākuta, rata
Drink	Inu
Medicine	Rongoa
Flu	Whurū
Germ	Iroriki
Health	Hauora, waiora, ora
Heart	Manawa, ngākau, whatumanawa, uho
Injection	Wero
Muscle	Uaua, io
Pregnant	Hapū
Urine/urinate	Mimi
Tongue	Arero
Chest	Poho
Breast (wai-u is breast milk)	Ū, uma, poho
Head	Upoko
Foot/feet/legs	Waewae
Eye	Karu/watu
Nose	Ihu
Ears	Taringa
Mouth	Waha/māngai
Arm(s)	Ringaringa
Hands	Ringaringa
Water	Wai
Fasting	Nohopuku
To treat	Rongoā

sysmex | Lighting the way with diagnostics

Fully Automated Urinalysis Workflow

UN-Series

Flexible

Modular and scalable, the UN-Series allows you to configure a complete urinalysis solution that best suits your lab environment and size, with the complete freedom to up- and down-scale.

Innovative

Leading-edge fluorescence flow cytometry means the UN-Series provides consistent and specific particle differentiation allowing your lab to analyse urine and body fluids with accuracy, precision and speed.

Intelligent

The UN-Series delivers full automation analysis, significantly reducing manual review and letting staff set up samples and walk away, increasing your lab's productivity.

Contact Sysmex to find out more about the UN-Series and how your lab could benefit from the flexible, innovative and intelligent urinalysis solution.

Colin McKenzie | E: mckenzie.colin@sysmex.co.nz | M: +64 (21) 625272

www.sysmex.co.nz