3 75th Anniversary Issue

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In this issue

How medical researchers conduct, and report sex and gender are under the spotlight in the editorial, advocating implementation of SAGER guidelines by journals to influence sex and gender-equity and sensitivity in the conduct of research and its publication.

Julie Creighton (Canterbury Health Laboratories) compares Liofilchem and Etest gradients strips and BD Phoenix in determining Vancomycin (MIC) of *Staphylococcus aureus* (MRSA). Isolates were tested for vancomycin susceptibility using the three methods and found considerable variation of results between methods and conflict amongst previously published studies. Vancomycin MIC was found to be method and user dependent and reporting of MIC at the single dilution level was discouraged, but to be used as part of clinical decision bundle for individualised therapy for serious MRSA infections.

Mycoplasma pneumoniae, Chlamydia pneumoniae, and *Legionella pneumophila* have a role in Community Acquired Pneumonia (CAP) establishment worldwide. Wael Al-Zereini from Mutah University in Jordan studied the utility of PCR and ELISA methods to determine prevalence of these pathogens in blood sera and nasopharyngeal samples detection of acute infection and their role as etiological agents in CAP patients. Results showed seroprevalence of the three atypical pathogens in patients was 64%, lower than reported CAP cases worldwide but comparable to other published rates and *C. pneumoniae* was the most likely agent for CAP in the Southern Jordan. PCR results could be used (with caution in single samples) simultaneously with serological methods to detect acute infection, improve the clinical utility and rapid diagnosis of CAP infections.

Richard Chen and colleagues from SCL Dunedin investigated the influence of storage time on the stability of routine coagulation parameters (INR, APTT and fibrinogen) at room temperature, in an effort to extend the maximum acceptable age of sodium-citrated specimens when there are delays in transport and storage across Otago/Southland due to adverse weather conditions. Results concluded that the maximum allowable age of uncentrifuged specimens for INR and fibrinogen could be extended to 72 hours at room temperature. However, APTT specimen age could not be changed. Centrifuged specimens allowed extension of ages for INR for up to 72 hours and fibrinogen for up to 96 hours.

Testing for selected inflammatory and haemolytic indicators were performed on 68 steady-state and asymptomatic sickle cell anaemia adolescents in Nigeria by Euphoria Akwiwu *et al.* at the University of Calabar. Leucocyte counts and bilirubin mean values were significantly higher while neutrophil and glutathione mean values were lower amongst the subjects living with sickle-cell anaemia compared to control subjects and these derangements were heightened by malaria infection. Reduced levels of glutathione and high bilirubin concentrations were indicative of oxidative stress and resulting inflammation typically associated with both sickle-cell anaemia and malaria and have a negative impact on the immune response and is a target of intervention for patients living with sickle-cell anaemia in malaria-endemic regions.

Zaki, Youness *et al.* investigated new diagnostic biomarkers; Cyclophilin A (CYPA) and Netrin-1 in Egyptian children with celiac disease, and age and sex matched healthy controls to determine their efficiency in the diagnosis and clinical follow up of celiac disease. Diagnosis was based on clinical features, biochemical investigations and upper endoscopy assessment. Serum CYPA and Netrin-1 and anti-tissue transglutaminase antibody levels were determined by ELISA. Results showed increased mean levels of CYPA and Netrin-1 compared to controls, demonstrating potential efficacy in diagnosis and follow up of celiac disease.

Paul Austin *et al.* conducted a retrospective study of N-methyl-D-aspartate glutamate receptor (NMDAR) antibody at LabPlus to determine if improvements immediately after aggressive treatment for anti-NMDAR encephalitis were retained consistently over time, could characterise the testing population, and establish overall performance of diagnosis and exclusion utility of the assay. In 2015, LabPlus launched a flagship demand-managed neuroimmunology diagnostic service, testing for NMDAR using qualitative Euroimmun Glutamate Receptor 3 IIF assay methods. Results demonstrated that the laboratory consistently met clinically required KPI for reporting TAT and delivered high value clinical results (antibody positive and negative), allowing rapid treatment intervention for patients with NMDAR encephalitis. The benefit of pre-analytical vetting of requests, neurologist experience, highly trained medical laboratory scientist and immunologist support, strong networks throughout the country's neurology teams, and high compliance for CSF specimen collection, contribute to this highly successful neuroimmunology diagnostic service.

External quality assessment (EQA) programmes are a critical aspect of laboratory quality management. The Pacific Pathology Training Centre (PPTC) provides the PPTC-EQA Programme to 86 participating laboratories in 22 countries in the Pacific region. Navin Karan *et al.* at PPTC describe programme funding, scheduling, shipping, and sample preparation for the molecular pathology disciplines and report overall performance improvement across all disciplines over the last five years.

Lin *et al.* from the Prince of Wales Hospital, NSW, present a case study on an anastomosing haemangioma benign vascular tumour identified in a rare location, namely a lymph node. This case study adds to the documented anatomical extent of this tumour and cautions against misinterpretation as a malignancy.

Chronic myeloid leukaemia (CML), BCR-ABL1 positive is a myeloproliferative neoplasm characterised by the reciprocal translocation of chromosome 9 and 22. While 95% of cases present in chronic phase and can be successfully treated and have near normal life expectancy, the accelerated or blast phase of the neoplasm are rare and often have an extremely poor clinical outcome. Jenny Marks (Waikato Hospital Laboratory) reports one such rare case, using fluorescence flow cytometry, CBC and blood films to show presence of over 20% blasts in peripheral blood and bone marrow. Up to half CML patients are asymptomatic and diagnosis can be a result of an incidental finding from routine blood work, so it is important to recognise persistence of neutrophilia and immature granulocytes or accompanying abnormalities such as eosinophilia or basophilia for early diagnosis and are key to ensuring prompt treatment and more favourable outcomes.

A Scientific Letter from Tara Feeley and Andrew Meisner report an apparent malfunction of the Thromboelastographic Haemostasis Analyser System (TEG®5000) and incompatibility with using Zoom telecommunications installed on the same PC to display the point-of-care thromboelastogram results.

We continue to celebrate the 75th Anniversary of the NZIMLS and the Journal with three member articles. Phillip Wakem and Ron Mackenzie reflect on the training and quality improvement programmes at the Pacific Pathology Training Centre, in Wellington over the last forty years. The evolution of haematology is discussed by Steve Johnson from MedLab Central with an eye to the future. Annette Bissett from Waitamata DHB and Ailsa Bunker from Middlemore Hospital, share their phlebotomist training, experiences and careers, from being an unrecognised profession to main-stream pre-analytical careers.

A two-year study conducted at Razi Hospital in Iran aimed to determine the microbiological characteristics of the highly prevalent bloodstream infections associated with in-dwelling intravascular and urinary devices such as catheters. These types of infections increase long-term hospitalisation, cost, morality, and mortality. 33% of tested catheters were positive for bacterial growth and antibiotic resistance patterns calculated for a total of 12 different types of equally Gram-positive and Gram-negative bacteria with a high rate of multi-drug resistance. These findings necessitate the implementation of more effective infection control policies and provide critical data for making better antibiotic selections, while recommending further multi-centre studies and larger sample sizes.

An evaluation of the effect of elevated serum level of the lipid indices on the alteration of erythrocyte indices was conducted in non-anaemic elderly people from the southwest of Iran. 275 healthy elderly people were assessed for the association between dyslipidaemia and circulating red blood cell indices and results showed statistically significant differences in non-anaemic elderly with high cholesterol, high triglycerides, and higher RBC counts, Hb and Hct, but not for MCV, compared to non-anaemic elderly with normal cholesterol and triglyceride levels.

Cardiac myosin-binding protein (cMYC) is a biomarker candidate for early diagnosis of acute myocardial infarction (AMI) due to its high expression in heart tissue. This marker is more abundant than the gold standard of AMI diagnosis; cardiac troponin (cTn). Masoumi *et al*. from Jundishapur University in Iran, examined changes in cMYC and cTn serum levels in AMI patients and report cMYC was significantly higher compared the control group and had a high diagnostic accuracy in the early hours since onset of symptoms, recommending further studies to confirm its utility as a predictive marker and complementary diagnostic test.

Our NZIMLS Council Technician Representative, Ajesh Joseph farewells Richard Ward, a long-standing member and well-regarded contributor to the medical laboratory profession.

The 4th year University of Otago BMLSc. students submit their Research Abstracts for Semester 1, 2021.

Lisa Cambridge
Deputy Editor

EDITORIAL

Recommendation to include sex and gender equity in research (SAGER) guidelines in research, publications and healthcare

Lisa Cambridge

There has been a groundswell of change from the traditional interpretation of sex and gender in society, science and the law over the last decade. More recently we have seen global opinion question the validity of sex hormone testing at the Olympics and in New Zealand, legislative amendments to the Birth, Deaths, Marriages and Relationship Registration Act (1995) will change the legal requirements for changing name or gender on birth certificates.

The terms sex and gender are inextricably linked and used interchangeably, but the two terms are not equivalent. Sex – refers to the biological attributes of either male or female based on their respective genitalia and reproductive function. Gender - describes the personal relationship with oneself, their body, their behaviours, social and cultural experiences. Traditionally, a new-born's sex is assigned as either male or female at birth based on the baby's genitals, it is then presumed that the child's gender will fall into one of these two roles, depending on society and cultural constructs. The LGBTQIA+ (Takatāpui) (lesbian, gay, bisexual, transgender (whakawāhine, tangata ira tane, fa'afafine and fakaleiti), queer, intersex, asexual) (1) community add complexity to these biological and social concepts.

Both sex and gender play roles in health and well-being, treatment availability and outcomes, environmental and occupational risks, even down to the way we seek health care (2). Biologically, we exhibit physiological differences in cardiovascular, respiratory, immunology, endocrinology, musculoskeletal and renal systems (3). The actions of pharmaceutical agents, marker expression, laboratory reference ranges (4), life span and disease prevalence also differ.

Chromosomal and genetic variations not only influence the differentiation of reproductive organs but also gene expression. Disorders of Sexual Development (DSD) (5) describes conditions ranging from significant (e.g., Intersex) to mild variations where development of sex chromosomes, gonad or sexual anatomy are considered, atypical. It has been postulated that as many as one in a hundred people have a form of DSD but may never know unless trying to conceive or being treated for another medical condition. Intersex people are born with sex chromosomes that signal as male or female, but the anatomy displays the other. Families of intersex babies are under enormous emotional pressure to "chose a sex" by undergoing surgery, giving no opportunity for the child to develop gender identity naturally (6). The right to reproduce and body integrity lawsuits have been brought and won against these surgeries (7).

Any binary view has been surpassed by a swathe of literature on the multidimensional concepts of sex and gender. It has been shown in clinical research that failure to separate each sex and gender-specific data, limits the power of data analysis and valuable study samples, result in adverse consequences, poor quality findings and data loss (8). At the coalface of healthcare, a lack of knowledge or prejudice by healthcare professionals result in assumptions of a patient's appearance and poor decisions on the use of binary-specific reference ranges.

How the science community, conduct and report medical research and operate within healthcare, matters. Heidari et al (9) referred to editors as "gatekeepers of science" influencing the conduct of research and its publication and advocating guidelines for clinical trial registration and ethical consent. In this respect, international journals drive transparency, equity and gender-sensitive reporting in research and internationally by implementing and adopting policies like the SAGER (Sex and Gender Equity in Research) Guidelines. Summarised in Table 1, these guidelines are the result of surveys conducted by the Gender Policy Committee, established by the European Association of Science Editors (EASE) in 2012. Surveys focused on four policy areas; instructions for authors to separate out sex and gender data, editorial board gender policies, gender balance amongst peer reviewers and provision of a tool for reviewers to assess and standardise sex-specific, sex as a biological variable, or gender-specific analysis and reporting in scientific publications.

Table 1: SAGER Guidelines for Research Publications

Principle	Recommendation
General	Correct use of the terms, sex and gender.
	Research elements should be designed and conducted in a way to enable analysis of sex and gender-related differences in the results, even if unexpected.
Title & Abstract	If only one sex or gender is to be included in the study, then the sex of any cells, tissues or other material derived from these should be specified, if appropriate.
Introduction	Report where relevant whether sex and/or gender differences may be expected, if appropriate.
Methods	Report if or how sex and gender were considered in the design of the study, whether they ensured adequate representation and justify any reasons for exclusion (e.g., cell lines).
Results	Data should be represented separated by sex and/or gender. Analysis should be reported regardless of positive or negative outcome. In clinical trials, data on withdrawals should also be separated by sex and gender.
Discussion	Potential implications of sex and gender should be discussed in the findings as they relate to the results. If sex and gender analysis was not conducted, the reason should be given and implications, limitation of lack of interpretation should discussed.

Implementing these steps would influence and guide the profession (10,11) across all areas of healthcare from improving sex-specific reference ranges for example, liver enzymes, creatinine, haemoglobin, iron studies and cardiac troponin (12) that are affected by sex hormones and body size as well as the heterogeneity seen in transgender populations affected by hormonal or surgical therapies. To taking detailed patient histories for most appropriate treatment and modifying laboratory test forms to include sex assigned at birth as well as gender, patients undergoing hormone therapy, or had surgery.

If editors are the gatekeepers of science and journals the voice, then surely the downstream effects of implementing such policies and guidelines into author instructions are ten-fold for research, healthcare and patients? Consideration of sex and gender differences and similarities lead the science community to conduct and publish, robust research and innovation in healthcare, with greater precision, sensitivity and relevant analysis for better outcomes in health and well-being.

But the real bottom line is human dignity, the belief that all people hold special value that is tied to their humanity and are worthy of respect regardless of age, ethnicity, status, gender, and sex.

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- 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

Comparison of Liofilchem and Etest gradient strips, and BD Phoenix, for the determination of vancomycin MIC in *Staphylococcus aureus*

Julie A Creighton

ABSTRACT

Introduction: Vancomcyin is the treatment of choice for serious infections caused by Gram-positive organisms, with therapy optimisation based on a calculation of the AUC_{24}/MIC_{BMD} ratio. Laboratories in New Zealand use a variety of methods to determine vancomycin MIC, including Vitek, Phoenix and Etest and Liofilchem MIC strips; however, there is a paucity of information on the performance of Liofilchem strips. This study compared Liofilchem and Etest gradient strips against Phoenix, for the determination of vancomycin MIC; also assessing variability between methods and operators to establish the reliability of reporting single dilution MIC values to clinicians.

Methods: A selection of 100 *Staphylococcus aureus* isolates, including 48 MRSA, were included in the study. Phoenix broth micro dilution (BMD), was performed using panel PMIC-84, and gradient strip MICs were performed using Etest and Liofilchem MIC Strips, recording single and double dilution MICs.

Results: All isolates were vancomycin susceptible, giving 100% categorical agreement. The essential agreement (EA) (MIC ±1 log_2) between all methods was 97%, with Phoenix and Etest showing the highest EA at 100% and modal values of 1.0 mg/L. Phoenix and Liofilchem had the lowest EA of 97%, due to the lower modal value of 0.5 mg/L produced by Liofilchem. Absolute agreement for single dilution values between Etest and Liofilchem was very low at 14%. Reader variability for the MIC strips ranged from 57% absolute agreement (Liofilchem at single dilution values) to 89% (Etest at double dilution values).

Conclusions: This study demonstrated high EA between methods, but considerable operator and method variation between MIC gradient strips. Liofilchem tended to produce MIC values one dilution lower than both Etest and Phoenix. These results have implications in terms of MIC method variability and the capacity of the laboratory to accurately report an absolute MIC result.

Key words: Liofilchem, Etest, Phoenix, MIC gradient strips, vancomycin, *Staphylococcus aureus.*

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INTRODUCTION

Vancomycin, a glycopeptide antibiotic which inhibits bacterial cell-wall synthesis, has been in use for the treatment of infections caused by Gram- positive organisms for over 60 years. Despite this extended period of use, decisions regarding optimisation of effective therapy, while avoiding toxicity, remain a problem for the treatment of serious infections such as bacteraemia.

The UK guidelines for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections have recently been updated (1). The review considered new evidence published over the last decade, new antimicrobials and changes in epidemiology in the UK. The use of vancomycin was still strongly recommended for the treatment of MRSA in serious infections such as bone and joint infections, severe cellulitis, bacteraemia and meningitis. While the guidelines discuss the use of trough serum concentrations to ensure non-toxic therapeutic levels of vancomycin, there is no mention of a laboratory input in terms of providing minimum inhibitory concentration (MIC) results to guide dosing.

In contrast, the Infectious Diseases Society of America (IDSA) therapeutic guidelines for serious MRSA infections, also published in 2020 (2), uses a different approach, looking to eliminate routine serum peak and trough concentration monitoring in favour of using a ratio of area-under-the-curve (AUC) over 24 hours to minimum inhibitory concentration (MIC) of ≥400. The AUC_{24}/MIC_{BMD} ratio ≥400 is based on the isolate MIC ≤ 1mg/L, as determined by reference broth microdilution (rBMD) method, in patients with normal renal function. However, the IDSA do not recommend waiting for the laboratory to complete vancomycin antimicrobial susceptibility testing (AST) before commencement of treatment because the AST report may take several days, laboratory testing methods vary and lack precision. Instead they advocate AUC-guided dosing, using limited serum sampling with Bayesian software interpretation, and assume a MIC_{BMD} of 1 mg/L (2).

So where does that leave the clinical laboratory in terms of testing and reporting vancomycin MICs, especially when several studies (3-5) have shown increased mortality or a worse clinical outcome in patients when the MIC is ≥1.5 mg/L? Can the laboratory determine an accurate and reliable MIC to the single or double dilution level when even the rBMD method has an acceptable variation of ±1 log_2 dilutions?

Using a rBMD method is time consuming and expensive and there are no breakpoints for disc diffusion testing, so most clinical laboratories in New Zealand use automated methods such as Phoenix or Vitek, or gradient MIC strips such as Etest or Liofilchem. Previous studies evaluating methods for the determination of vancomycin MIC have compared Vitek, Phoenix and Etest (3-5); however, there is a paucity of information on the performance of Liofilchem MIC strips. This study compared the performance of Liofilchem and Etest gradient strips, and $Phoenix_{BMD}$, for the determination of vancomycin MIC, against 100 clinical *Staphylococcus aureus* isolates, including 48 MRSA. The study also assessed the variability between operators and methods to determine the reliability of reporting single dilution MIC values to clinicians.

METHODS

The study consisted of a retrospective selection of 100 non-duplicate *Staphylococcus aureus* isolates, including 48 MRSA, collected between 2011 and 2021. All isolates were obtained from clinical samples, predominantly blood cultures and other sterile-site samples, which had been processed at Canterbury Health Laboratories. All isolates had a previous vancomycin susceptibility result determined by Phoenix (BD Diagnostics, USA) broth micro dilution (BMD), using panel PMIC-84. The BD -EpiCenter database was searched to selectively find isolates with an MIC of 2 mg/L, revealing 9 isolates which were included in the study. Isolates stored at -70°C were sub-cultured twice onto Colombia Blood Agar. *S. aureus* ATCC 29213 was used as a control strain.

Confirmation of isolate identification was performed by MALDI -TOF (Bruker Daltonics, USA). Gradient strip MICs were prospectively performed using Etest (bioMèrieux, France) and Liofilchem MIC Strip Test (Liofilchem, Italy), following the manufacturer's instructions, with interpretation according to EUCAST breakpoints. Gradient strips were independently read by three scientists, and the modal value was used as the consensus MIC. For the purposes of this evaluation, single dilution (0 $\log_2$) values were recorded as well as doubling dilution values.

RESULTS

One hundred non-duplicate *S. aureus* isolates were tested for vancomycin susceptibility using three different methods: Phoenix BMD, Etest gradient strips and Liofilchem MIC strip tests. The Phoenix provides MICs as doubling dilution concentrations (range ≤0.5 to >16 mg/L). MIC gradient strips are normally rounded up to the next doubling dilution value; however, for the purposes of this evaluation, MIC strip results were recorded at both the single and double dilution value.

Operator variability

The MIC strips were independently read by three scientists, with the modal value used as the consensus MIC for further method comparisons. Since interpretation of the MIC endpoint can be subjective and operator-dependent, the results were first assessed for variation between operators, at both the single and double dilution endpoints. For the Etest, at the single dilution reading, 77/100 (77%) of isolates had the same MIC value (absolute agreement) recorded by each of the three readers, with the remaining 23 isolates having matching MICs for two of the three readings (Table 1). When considering the doubling dilution results, 89% of the isolates had matching MICs, recorded by each of the three readers. Endpoint interpretation for the Liofilchem strip was less consistent, with only 57% of isolates recording the same MIC value for the single dilution results, by all three readers, increasing to 79% agreement for the double dilution values (Table 1). For both gradient strips, there were no isolates for which three different MIC endpoints were recorded. These results show good inter-operator concordance, confirming the use of the modal MIC for further analysis.

Table 1. Inter-operator consensus of three readers for vancomycin MIC of 100 *S. aureus*, determined by Etest and Liofilchem gradient strips.

AST method	Reader absolute agreement (%)	Two reader absolute agreement (%)
Etest		
single dilution	77	23
double dilution	89	11
Liofilchem		
single dilution	57	43
double dilution	79	21

Categorical and essential agreement

All isolates were categorised as vancomycin susceptible (MIC ≤ 2 mg/L) by all methods, giving 100% categorical agreement (CA). The essential agreement (EA) between methods, at the doubling dilution MIC value, was based on having no more than one concentration different from that reported by the other method (MIC ±1 $\log_2$). Overall, the EA between all methods was high at 97% (Table 2). Phoenix and Etest showed the highest EA at 100%, while Etest and Liofilchem showed 99% EA at the ±1 $\log_2$ level and 97% EA at the ±0 $\log_2$ level. Phoenix and Liofilchem had the lowest EA of 97%.

Table 2. Essential and absolute agreement between MIC methods for the determination of vancomycin MIC in 100 *S. aureus* isolates.

AST method	Essential agreement (%)	Absolute agreement (%)
All methods	97	42
Phoenix V Etest	100	89
Phoenix V Liofilchem	97	47
Liofilchem V Etest (1± dilution)	99	46
Liofilchem V Etest (0± dilution)	97	14

Absolute agreement

Considering absolute agreement (matching MIC values) between methods, at the doubling-dilution concentrations, there is greater variation, with an all-method agreement of only 42% (Table 2). The Phoenix and Etest showed the highest absolute agreement at 89%, while MIC values for Phoenix and Liofilchem agreed only 47% of the time. Comparing gradient strips Etest and Liofilchem, the absolute agreement was low at 46%. Strikingly only 11% of MICs were ≥1.0 mg/L by Liofilchem, whereas 76% of MICs were ≥1.0 mg/L by Etest. Comparing Etest with Liofilchem at the single dilution level, the MICs were in absolute agreement for only 14% of the isolates. Furthermore for three isolates the results differed by three one-fold dilution values: all with Etest MICs higher than Liofilchem.

Modal values and MIC scattergrams

The low level of agreement between Liofilchem MICs and those produced by Phoenix and Etest, can also be demonstrated by the different modal values and shown in the scattergrams of MIC results (Figure 1). The modal value for Phoenix was 1.0 mg/L, with 82/100 (82%) of isolates having this MIC, with an additional 9 isolates having an MIC of 0.5 mg/L and the remaining 9 isolates having an MIC of 2 mg/L. At the doubling dilution concentrations, the Etest MICs aligned closely with the Phoenix results, producing a modal value of 1.0 mg/L, with 85% of isolates having this MIC, 6 isolates with an MIC of 0.5 mg/L and the remaining 9 isolates having an MIC of 2 mg/L. In contrast, Liofilchem had a modal value of 0.5 mg/L, although that comprised only 52% of isolates, with the remaining 48% producing an MIC of 1.0 mg/L.

There were 11 isolates which produced an MIC >1 mg/L by any method, only one of which was a MRSA. Seven of these isolates were detected by both Phoenix and Etest, with an additional two isolates detected by each system; however, for Liofilchem there were no isolates which had an MIC >1 mg/L (Figure 1).

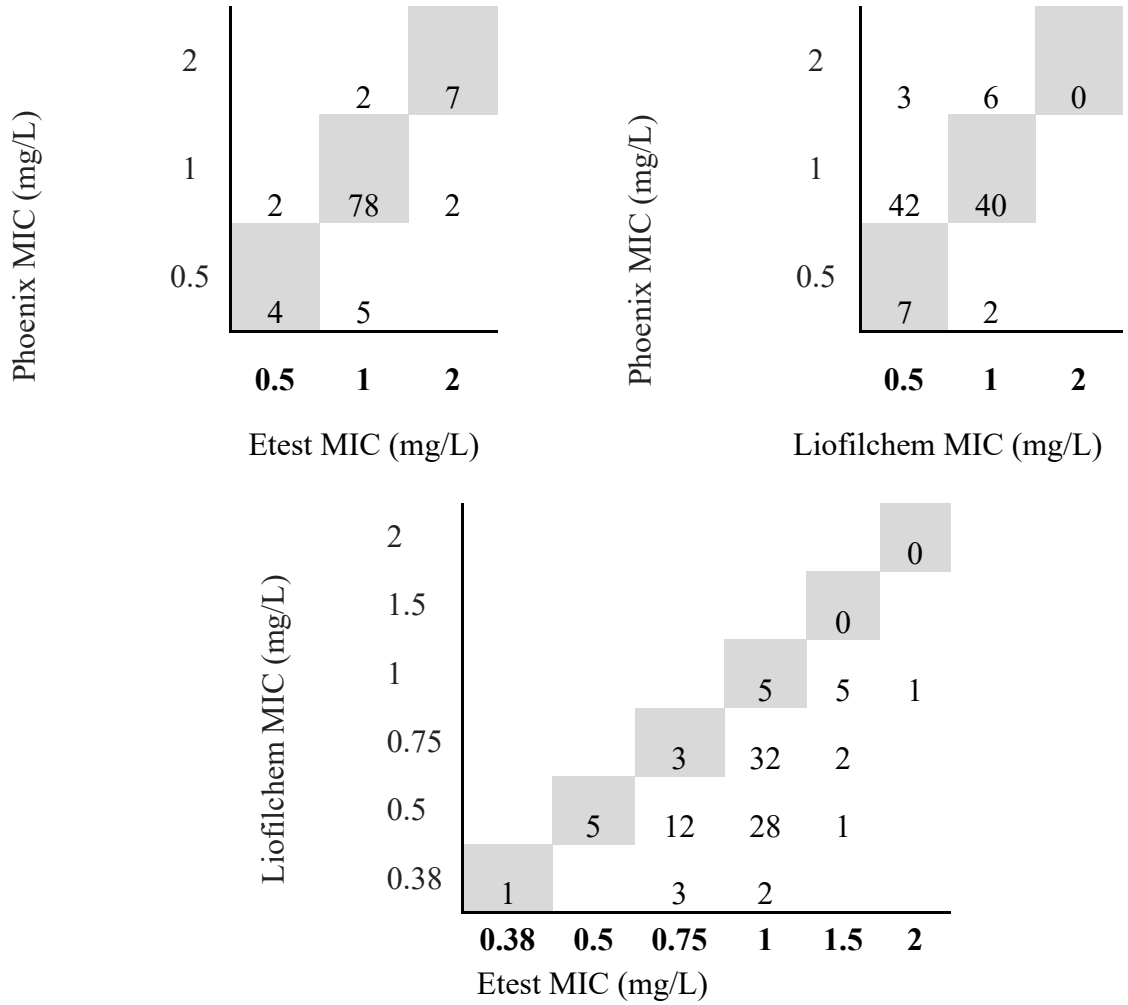

Figure 1. Scattergrams of MIC results (mg/L) for 100 *S. aureus*, comparing Phoenix with Etest, Phoenix with Liofilchem and Liofilchem with Etest.

DISCUSSION

This study was performed to evaluate vancomycin Liofilchem MIC strips and to assess the reliability of reporting single dilution MIC values. We found 100% CA and high EA, at doubling dilution MICs, between all methods for the determination of vancomycin MIC against 100 *S. aureus* (48 MRSA) isolates. However, if a laboratory is required to produce a vancomycin MIC result for a clinician's AUC_{24}/MIC_{BMD} calculation, the MIC level should be accurate and reproducible, so that small changes in the MIC do not have a dramatic effect on the dose given, nor initiate an unnecessary switch in treatment options.

We have demonstrated considerable variation between methods, ranging as low as 14% absolute agreement between Etest and Liofilchem at the single-dilution concentration. When MIC values were rounded up to the next doubling dilution, the absolute agreement between all methods was still poor. Phoenix and Etest results aligned most closely for absolute agreement and essential agreement. Furthermore, both produced a modal value of 1 mg/L and had similar MIC distributions for the study isolates. On the other hand, Liofilchem showed discordant absolute agreement between both Phoenix and Etest, frequently producing MICs 1 $\log_2$ concentration lower, with a modal value of 0.5 mg/L.

It is interesting that our study has shown such a high level of concordance between Phoenix and Etest. This finding conflicts with previous studies which have found that Etest tends to produce MIC values 1 to 2 dilutions higher than reference BMD (rBMD) and that Phoenix produces MICs 1 dilution lower (4,6). In a study by Rybak *et al*. evaluating the ability of Etest, Microscan, Vitek 2 and Phoenix to determine the vancomycin MIC of 200 MRSA isolates, the authors found that the Phoenix achieved 66.2% absolute agreement with rBMD. On the other hand, Etest achieved only 36.7% agreement, with the low rate attributed to Etest producing MIC values 1 to 2 dilutions higher than rBMD. Rybak suggested that Etest might be a conservative approach to determining vancomycin MIC, especially in patients with serious infections (4). An investigation by Riedel *et al*. of 150 *S. aureus* isolates (100 MRSA), comparing Microscan, Phoenix and Etest with rBMD (6), showed similar findings in that the modal MIC for rBMD, Microscan and Etest was 1.0 mg/L, with Etest commonly producing MICs 1 $\log_2$ concentration higher than rBMD; while Phoenix produced MICs 1 $\log_2$ lower and a modal MIC of 0.5 mg/L (6).

Contrary to these studies, a recent review by Brusamarello *et al*. (7) found a high rate of variability between rBMD and Etest, ranging from 0% to 89%. Moreover, in five of six studies vancomycin MICs determined by Etest were typically either concordant with, or lower than, rBMD. Collectively, these conflicting findings between methods would suggest that establishing a true vancomycin MIC is highly method and user dependent.

The difference between our study and others might be explained by different Phoenix panel types; however, our institution has been using the Phoenix for over 10 years, with several different panel types, testing over 54,000 *S. aureus* isolates. During this period 85% of isolates had an MIC of 1.0 mg/L, 16.6% had an MIC of 0.5 mg/L and 0.6% had an MIC of 2 mg/L, demonstrating a consistency of results. Differences in MIC gradient strip values might be attributed to reader subjectivity or institutional interpretation of endpoint cut-off (8).

MIC endpoint interpretation is important as studies investigating treatment outcomes in relation to MICs have found an association between a high vancomycin MIC and treatment failure (3,5). Van Hal *et al*. conducted a meta-analysis of 22 studies which reported on vancomycin treatment outcomes for MRSA infections. The authors concluded that a high vancomycin MIC of ≥2 mg/L was a predictor of treatment failure, and a higher mortality was associated with MRSA blood stream infections if the vancomycin Etest was ≥1.5 mg/L. Their analysis found Etest results to be 0.5 to 1 dilution higher than rBMD and recommended its use to detect patients potentially at risk for treatment failure (3). Similarly, Chen *et al*. retrospectively reviewed over 300 patients with MRSA bacteraemia who were treated with vancomycin, finding that high MICs by Etest was an independent predictor of patient mortality (5).

However, there are many opposing voices arguing against attributing treatment failures based on a single MIC value. A robust meta-analysis by Dalton *et al*., emphasising treatment failure and mortality outcomes, showed that differences in AUC and MIC methodology, combined with varying patient co-morbidities and sites of infection, made it difficult to compare studies (9). The authors failed to find a meaningful relationship between AUC/MIC and predication of clinical outcome.

More importantly is the premise that routine laboratories can provide an accurate and reproducible MIC value that can be reliably used in the AUC_{24}/MIC_{BMD} calculation to guide dosing (8). Indeed, laboratory MIC determination is fraught with difficulties including method variations, strain heterogenicity, operator interpretation and inter-laboratory differences (8). Even within the accepted MIC variation of ±1 $\log_2$ dilutions, a single MIC value could be 0.5 mg/L or 2 mg/L on any subsequent repeat test. Therefore, even a small over estimation of MIC could initiate a change in therapy or potentially result in patient toxicity if the dosage is adjusted accordingly upwards (7). It might be that other variables, such as the site or underlying cause of infection together with patient co-morbidities, play a greater role in determining patient outcomes rather than isolate MIC. Further research into the treatment of MRSA bacteraemia, using alternative options such as daptomycin combined with beta-lactams, may offer a better pathway for clinicians (10).

Limitations

This study has some limitations including no comparison against the reference BMD method, the isolates were obtained from only one institution and clinical outcomes in relation to MIC values were not investigated.

In summary, this study has found total categorical agreement, and a high level of essential agreement at doubling dilution concentrations, for vancomycin susceptibility testing between Phoenix, Etest and Liofilchem. In addition, there was high absolute concordance between Etest and Phoenix, with both yielding a modal value of 1.0 mg/L and having a similar distribution of MIC results. However, there was considerable variation between Liofilchem and Etest, with very low absolute agreement. This study observed that the Liofilchem MIC gradient strips produced MIC values one dilution lower than both Etest and Phoenix. This result has implications in New Zealand as many laboratories use the Liofilchem vancomycin MIC strips and they may be unaware that this product may undercall MIC values.

The results of this study call into doubt the ability of the laboratory to accurately produce an absolute MIC at any level better than a categorical result. Reporting of MICs at the single dilution level is highly discouraged. In general, clinicians should assume a vancomycin MIC of 1 mg/L ±1 $\log_2$ dilutions. However, as part of individualised therapy in patients who have serious MRSA infections, the laboratory MIC result could be reported as part of the clinical decision bundle, contingent with the clinician's understanding of MIC test variability and the laboratory method used.

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ORIGINAL ARTICLE

Simultaneous detection of *Chlamydia pneumoniae*, *Mycoplasma pneumoniae,* and *Legionella pneumophila* in patients hospitalised for community-acquired pneumonia in South Jordan

Wael A Al-Zereini

ABSTRACT

Objectives: To detect *Chlamydia pneumoniae, Mycoplasma pneumoniae,* and *Legionella pneumophila* simultaneously in hospitalized community-acquired pneumonia (CAP) patients in Southern Jordan; the diagnostic utility of PCR and ELISA methods in determining their prevalence, detection of acute infection and identification of the causal agent from a single serum.

Methods: Blood sera and nasopharyngeal samples were collected from 200 participants (100 individuals from each of CAP patients and controls). Seroprevalences of IgG and IgM antibodies raised against the three pathogens was analysed in collected sera by ELISA, while presence of their DNA in nasopharyngeal samples was detected by standard PCR. Concurrent infection was detected by multiplex PCR.

Results: Based on ELISA-IgG, the general prevalence rates of *C. pneumoniae* and *M. pneumoniae* were significantly higher in CAP cases than controls (p= 0.02 and $p\leq$ 0.001, respectively); anti-*L. pneumophila* IgG was not detected in all participants. Based on ELISA-IgM and PCR in detecting acute infections, significant higher detection frequencies of anti-*C. pneumoniae* IgM and DNA were noticed in CAP patients compared to control cohort (p= 0.01 and $p\leq$ 0.001, respectively); an insignificant difference in prevalence rates of *M. pneumoniae* and *L. pneumophila* between patients and controls were reported in both assays. Concurrent detection of the three pathogens was noticed in 30% of entire CAP cases.

Conclusions: Simultaneous use of ELISA and PCR assays may allow rapidity and improvement in detection of CAP etiology in acute diseases; *C. pneumoniae* is the most possible etiological agent for CAP in Southern Jordan population during the study period.

Keywords: *Chlamydia pneumoniae*, *Mycoplasma pneumoniae, Legionella pneumophila,* enzyme-linked immunosorbent assay (ELSA), polymerase chain reaction (PCR), Jordan.

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INTRODUCTION

Community acquired pneumonia (CAP) is a respiratory infection that causes morbidity and mortality worldwide. Atypical pathogens such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae,* and *Legionella pneumophila* have a role in CAP establishment. They account for 22% of CAP worldwide (1,2) and 40%-60% of hospitalised patients (3,4). *C. pneumoniae* infections are evident in about 1%-22% of all pneumonia cases; *M. pneumonia* in 8%-30% which might reach to 50% in outbreaks, and *Legionella* species caused pneumonia in 2%-9% of patients (2,5,6). Respiratory infections due to these pathogens have similar clinical features and their isolation and culturing require complex procedures, rendering it difficult managing pneumonia patients and the rapid detection of etiological agents. Furthermore, false positive cross reactions may occur in patients infected with other bacteria due to delayed or abated immune response (7). As more than 50% of CAP cases are treated with antibiotics based on the most likely causing pathogen without identifying the main etiological agent, it is necessary to have approaches that can detect and differentiate these respiratory pathogens using the same sample and the same assay.

Several previous studies in Jordan have highlighted the prevalence of these bacteria by serological detection of immunoglobulins IgG and/or IgM using micro-immunofluorescence (MIF) as a gold standard diagnostic method, or ELISA assays alone or with PCR. Serological studies revealed prevalence of *C. pneumoniae* in 54.4%-61% of apparently healthy individuals (8,9) and in 23%-70% of adult patients with respiratory tract infections (10,11). Antibodies raised against *L. pneumophila* and *M. pneumoniae* were noticed in 6% (11) and 7%-8% of CAP patients (12), respectively.

However, the presence of *C. pneumoniae* DNA was evident from 8.8% of adult CAP cases (11) and 4.5% of children (13) using PCR assay; PCR failed to detect the three pathogens in children younger than 2 years old (14) and *M. pneumoniae* in nasopharyngeal samples from adult patients (12). All the aforementioned studies were carried out in central and north regions of Jordan; a large part of these did not include controls to establish a baseline of seropositivity. In a recent study performed in Southern Jordan, prevalence of *C. pneumoniae* was reported in 44.3%, 27%, and 40% of hospitalised CAP individuals based on IgG, IgM, and PCR respectively (6); the authors did not consider the concurrent infection with other atypical pathogens.

The current study is the first that address simultaneous detection of *C. pneumoniae, M. pneumoniae,* and *L. pneumophila* with evaluating their prevalence in hospitalised CAP patients from Southern Jordan, especially from Al-Karak Governorate. This was assessed using ELISA and PCR assays as rapid techniques; ELISA-IgG was employed to detect the presumptive role of each pathogen in CAP occurrence or in past-infections while both ELISA-IgM and PCR were used in revealing recent (current) infection by these bacteria.

MATERIAL AND METHODS

Study subjects and sample collection

The study was performed from January 2016 to December 2017 and included 100 CAP patients and 100 asymptomatic controls. Patients were those hospitalized in Al-Karak Governmental Hospital with a clinical and radiological diagnosis of CAP (i.e. fever, sputum production, cough, dyspnea, pulmonary infiltrates on chest x-ray, etc.).

Their demographics, comorbidities, and clinical data were documented. Selected control subjects were asymptomatic blood donors, laboratory personnel, and co-workers at Al-Karak Hospital. Control participants did not have respiratory diseases or took antibiotics during the three months preceded their enrolment in this study. CAP patients and controls were divided into four age groups: 17–32 years, 33–48 years, 49–64 years, and ≥65 years.

Clinical samples (blood and nasopharyngeal swabs) from CAP patients were collected within 48 h of hospital admission. Venous blood samples (3-4 ml each) were withdrawn from all participants into gel-containing plain tubes (AMPulab™, Germany). Clotted blood samples were centrifuged at 3,500 rpm for 10 minutes (Combi-514R, South Korea) and the resulted sera were placed in sterile Eppendorf tubes; they were stored at -20 °C and used to detect the antibodies raised specific against each pathogen. However, nasopharyngeal specimens collected by sterile plastic-shafted Dacron-tipped swabs were immediately placed in a sterile screw-capped tubes containing 1 ml of transport medium (Vircell Microbiologists, Spain) and stored at -70°C until analysed for presence of the pathogens' DNA.

The Scientific Research Committees at the Department of Biological Sciences, Faculty of Scientific Research, and the Scientific Ethics Committee at Department of Medicine, Mutah University-Al-Karak, Jordan, approved the study (no. 201514). The research was performed in accordance with the Helsinki declaration.

Immunoglobulin G (IgG) and immunoglobulin M (IgM) detection by ELISA

Anti-*C. pneumoniae,* anti-*L. pneumophila,* and anti-*M. pneumoniae* IgG and IgM antibodies were detected using commercial kits (Vircell Microbiologists, Spain) and following manufacturer's instructions. Detection was performed spectrophotometric using microplate reader (BioTek ELx800, South Korea) at $A_{450/630\ nm}$. The IgG sorbent was added to each sample well in IgM antibody detection assays to avoid false positive results due to rheumatoid factor and false negative results due to an excess of IgG antibodies.

Nucleic acid extraction and PCR assays

DNA was extracted from collected nasopharyngeal samples using G-spin™ total DNA Extraction kit (iNtRON Biotechnology, Korea). DNA concentration and purity was determined spectrophotometric at $A_{260/280nm}$. The forward and reverse primers (Midland Company Inc., USA) used in the PCR assay were from literature (4) targeting the *C. pneumoniae* specific *Pst1* fragment, the macrophage infectivity potentiator gene (*mip*) of *L. pneumophila,* and the P1 cytadhesion of *M. pneumoniae*. The amplification reaction in single PCR contained 300 ng of extracted DNA, 10 µl of 2x PCR master mix solution (i-Max II, iNtRON Biotechnology, Korea), 1.5 µl of each forward and reverse primer (10 pmol/µl) and completed with nuclease-free water to 20 µl. Whilst, multiplex PCR reaction contained 500 ng of template DNA, 10 µl of 2x master mix (MultiMAX, iNtRON Biotechnology, Korea), 6 µl of primer mixture (10 pmol/µl) and completed with nuclease-free water to 20 µl; primer mixture included 1.5 µl from each forward and reverse *pst1*, *mip* and P1 primers.

The cycling conditions were as follow: denaturation for 5 minutes at 95 °C followed by 45 amplification cycles. Each cycle consisted of a denaturation step for 1 minute at 95 °C, annealing for 1 minute at 50 °C (single PCR) or 55°C (multiplex PCR), extension for 1 minute at 72 °C and a final elongation step for 7 minutes at 72 °C. In every PCR run, a negative (nuclease-free water) and positive (AMPLIRUN® *Chlamydophila pneumonia* DNA, AMPLIRUN® *Legionella pneumophila* DNA, and AMPLIRUN® *Mycoplasma pneumoniae* DNA) (Vircell Microbiologists, Spain) controls were used instead of the DNA harvested from the clinical specimens. Amplification products were analyzed by 1.5% agarose gel electrophoresis at 5 $V.cm^{-1}$ and visualized under UV.

Criteria for etiological diagnosis

Patients eligible for inclusion in the current study were those with symptoms and signs of fever (temperature ≥38°C), sputum production, cough, chest pain, and dyspnea. They fulfilled the definition of CAP and the presence of new pulmonary infiltrates on chest x-ray confirmed the diagnosis. Patients with nosocomial pneumonia, active tuberculosis, or discharged from hospital a month prior their current hospitalisation due to pneumonia were excluded.

According to the Vircell kits' instructions, IgG or IgM arbitrary index >11 was considered as a serological marker for seropositivity. IgG was regarded as an indication on presumptive bacterial infection, whilst detection of IgM in sera or bacterial DNA in nasopharyngeal samples were indicative of a current (acute) infection.

Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences software, version 14.0.1 (SPSS Inc., Chicago, USA). Calculation of the specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) was performed for ELISA-IgM and PCR tests in CAP cases with acute infection. Multiple correlation coefficients were calculated for all methods used in infection detection among CAP patients. Data were statistically analysed using Chi-squared test ($\chi 2$) and Fisher's exact test. P <0.05 was considered statistically significant.

RESULTS

One hundred from each of hospitalised CAP patients (53 males and 47 females, mean age 45.3±23.5 years) and asymptomatic controls (51 males and 49 females, mean age 44.5 ± 13.9 years) were included in the current study. All CAP cases have fulfilled the inclusion criteria. Insignificant statistical differences were detected between the mean ages of CAP cases and controls (p=0.28) as well as between individual numbers from each gender in both cohorts (p=0.94), indicating almost age and sex matching between controls and patients.

Overall seroprevalence of *C. pneumoniae*, *M. pneumoniae*, and *L. pneumophila* in controls and cases

The general prevalence of the three pathogens in CAP and control cohorts was assessed by detecting the anti-pathogen specific IgG antibodies in collected sera (Table 1). A significant 1.6 times higher prevalence of anti-*C. pneumoniae* IgG was observed in CAP cases than in control group (40% vs 25%, p= 0.02). The highest detection frequency of the antibody was noticed in controls age group ≥65 years (37.5%), whilst it was > 80% in CAP age groups over 48 years. Overall seroprevalence of *M. pneumoniae* IgG was almost four times higher in CAP patients compared to controls (22% vs 5%, p= < 0.001); it was the highest at age group (49-64 years, 30%) in CAP cases and in controls age bracket (≥65 years, 12.5%). Noticeably, anti-*L. pneumophila* IgG was not detected in both patients and controls.

Detection rate of immunoglobulin M (IgM) in controls and CAP patients

Anti-*C. pneumoniae* IgM was detected at a significantly higher rate (2.5 times) in CAP patients compared to the control cohort (19% vs 7%, p=0.01); the detection rate also increased with age in the CAP groups and reached a peak of 100% in the age bracket ≥65 years). In the control group, the highest IgM seroprevalence was reported in the age group 33-48 years (13.6%). However, an insignificant difference was noticed in anti-*M. pneumoniae* IgM between the patient and control groups (6% vs 3%, p=0.25); it was detected in one age group of controls (17-32 years) and in two patients' age groups (33-48 years and 49-64 years). Intriguingly, anti-*L. pneumophila* IgM was absent in just one CAP cases age group (≥65 years); its seroprevalence was statistically insignificant between the two included cohorts (11% vs 14%, p=0.3) (Table 2).

Table 1. Seroprevalence of anti-*C. pneumonia,* anti-*M. pneumoniae* and anti-*L. pneumophila* based on detection of IgG antibodies in patients and controls and their age-wise distribution.

Age group (years)	Number positive (% positivity)		*P**
	Controls (n=100)	Patients (n=100)	
C. pneumonia			
17-32	12/55 (21.8)	21/62 (33.9)	0.11
33-48	6/22 (27.3)	6/23 (26.1)	0.5
49-64	4/15 (26.7)	8/10 (80)	0.01
≥65	3/8 (37.5)	5/5 (100)	0.04
Total positive	25 (25)	40 (40)	0.02
M. pneumoniae			
17-32	1/55 (1.8)	14/62 (22.6)	< 0.001
33-48	2/22 (9.1)	5/23 (21.7)	0.2
49-64	1/15 (6.7)	3/10 (30)	0.16
≥65	1/8 (12.5)	0/5 (0)	0.6
Total positive	5 (5)	22 (22)	< 0.001
L. pneumophila	ND	ND	-

* Statistically significant differences compared to the control group ($P<0.05$). ND: not detected.

Table 2. Age distribution of anti-*C. pneumoniae,* anti-*M. pneumoniae* and anti-*L. pneumophila* IgM antibodies and nasopharyngeal PCR positivity for determination of acute infections in CAP patients and controls.

Age group (years)	Number positive (% positivity)					
	IgM		*P**	PCR		*P**
	Controls (n=100)	Patients (n=100)		Controls (n=100)	Patients (n=100)	
C. pneumonia						
17-32	3/55 (5.4)	9/62 (14.5)	0.09	12/55 (21.8)	23/62 (37.1)	0.05
33-48	3/22 (13.6)	3/23 (13)	0.6	3/22 (13.6)	11/23 (47.8)	0.01
49-64	1/15 (6.7)	2/10 (20)	0.3	4/15 (26.7)	6/10 (60)	0.1
≥65	0/8 (0)	5/5 (100)	< 0.001	1/8 (3)	3/5 (60)	0.1
Total positive	7 (7)	19 (19)	0.01	20 (20)	43 (43)	< 0.001
M. pneumoniae				ND	ND	-
17-32	3/55 (5.4)	0/62 (0)	0.1			
33-48	0/22 (0)	5/23 (21.7)	0.03			
49-64	0/15 (0)	1/10 (10)	0.4			
≥65	0/8 (0)	0/5 (0)	1			
Total positive	3 (3)	6 (6)	0.25			
L. pneumophila						
17-32	4/55 (7.2)	8/62 (12.9)	0.2	1/55 (1.8)	4/62 (6.5)	0.2
33-48	4/22 (18.2)	5/23 (21.7)	0.5	2/22 (9.1)	2/23 (8.7)	0.7
49-64	1/15 (6.7)	1/10 (10)	0.6	1/15 (6.7)	1/10 (10)	0.6
≥65	2/8 (25)	0/5 (0)	0.4	0/8 (0)	0/5 (0)	1
Total positive	11 (11)	14 (14)	0.3	4 (4)	7 (7)	0.3

Detec tion of nucleic acid by PCR for the pathogens

Nasopharyngeal samples from enrolled subjects were tested for the presence of each pathogen's DNA as a criterion of recent infection by conventional PCR; multiplex PCR was performed on all samples to reveal concurrent detection of more than one pathogen-specific DNA in a single sample (Figure 1). Optimized PCR condition in both conventional and multiplex assays gave the same outcomes. Surprisingly, PCR failed to detect *M. pneumonia* DNA in all tested nasopharyngeal samples; it was less efficient in amplifying the DNA of positive control (well no# 4). *C. pneumoniae* DNA was detected in all age groups of controls and CAP participants; the detection rate was significantly 2.15 folds higher in CAP cases than controls

(43% vs 20%, $p< 0.001$). An insignificant difference was noticed in detection rate of *L. pneumophila* DNA between patient and control individuals (7% and 4% respectively, $p=0.3$) with absence of the bacterium nucleic acid in age group ≥65 years of both cohorts (Table 2). Overall, these data verify the results of IgM that relate between *C. pneumoniae* and CAP in Southern Jordan. Noteworthy, all nasopharyngeal samples of CAP cases and 75% of controls that were positive to *L. pneumophila* DNA were also positive to *C. pneumoniae* in multiplex PCR.

Comparison between IgM antibody and PCR in detection of acute infection with correlation of ELISA-IgM and molecular results

In the entire CAP patients, 19 individuals demonstrated seropositivity to anti-*C. pneumoniae* IgM and 43 nasopharyngeal samples revealed presence of *C. pneumoniae* DNA. Meanwhile, anti-*M. pneumoniae* IgM was detected in six patients with absence of its DNA in the nasopharyngeal samples; *L. pneumophila* was identified in 14 and seven CAP cases based on IgM and PCR, respectively. In controls, seven sera were positive to anti-*C. pneumoniae* IgM and 20 nasopharyngeal samples demonstrated the presence of the bacterial DNA. Furthermore, three sera were anti-*M. pneumoniae* IgM positive with PCR being less efficient in detecting bacterial DNA in the nasopharyngeal samples; anti-*L. pneumophila* IgM and bacterium nucleic acid were evident in 11 sera and four nasopharyngeal samples, respectively, indicating a cross reactivity in ELISA assay and false-positive PCR results as asymptomatic carrier of *L. pneumophila* is not recognised. Considering ELISA-IgG seropositive results in CAP patients, 45% (18/40) and 22.5% (9/40) of *C. pneumoniae* positive cases were PCR- and IgM-positive, respectively; all of *M. pneumoniae* positive persons were PCR negative and 18% (4/22) revealed positivity to IgM (Figure 2). However, in asymptomatic controls, 4% (1/25) and 48% (12/25) of individuals with seropositivity to *C. pneumoniae* IgG were positive to IgM and PCR, respectively. Remarkably, none of the controls carried *M. pneumoniae* DNA in the respiratory tract.

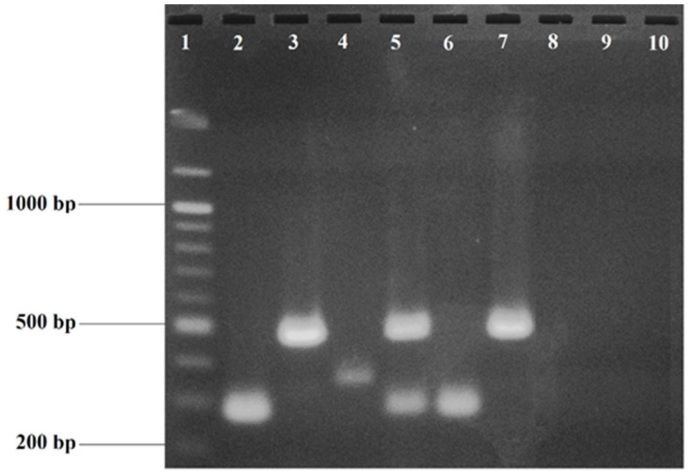

Figure 1. Gel-electrophoresis revealing the amplification of atypical pathogenic bacteria, single PCR for positive controls and multiplex PCR for examples from CAP cases. Lane 1: molecular size DNA ladder 100 bp, lanes 2: single PCR reaction for *Pst1* fragment of *C. pneumoniae* (283 bp), lanes 3: single PCR reaction for *mip* fragment of *L. pneumonophila* (487 bp) and lane 4: single PCR reaction for P1 fragment of *M. pneumoniae* (360 bp). Multiplex PCR of genes combination, lane 5: sample # 2; lane 6: sample # 10 and lanes 7: sample # 5. *M. pneumoniae* DNA was not detected in CAP or control samples.

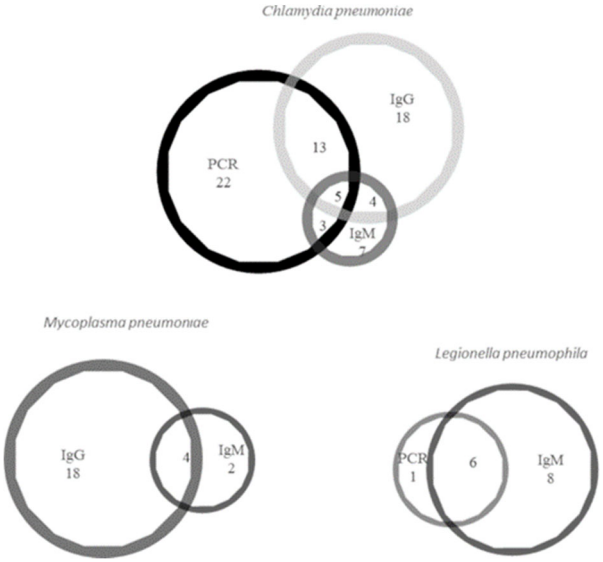

Figure 2. Venn diagram revealing the relation between diagnostic tests employed for detection of pneumonia infection in CAP patients.

All these data indicated a strong correlation between PCR and both ELISA-IgG and –IgM (r = 0.74 and 0.88, respectively) but a very week correlation between ELISA-IgG and –IgM (r = 0.33) in detection of infections in CAP patients. Furthermore, PPV, NPV, sensitivities, and specificities of both assays were calculated for diagnosis of acute infections using diagnosed CAP cases as the gold standard (Table 3). ELISA-IgM was less sensitive but more specific than PCR in detecting *C. pneumoniae* infections (19% vs 43% and 93% vs 80%, respectively); it has improved PPV over PCR (73.08% over 68.25%) as indicative on acute infections. PCR and IgM were highly specific to *M. pneumoniae* (97%-100%) with a very low sensitivity (0-6%); meanwhile, IgM was more sensitive than PCR in detecting *L. pneumophila* with a comparable specificity. Worth noting, PCR had high PPV than anti-*L. pneumophila* IgM as indicative on occurrence of acute infections.

Table 3. Sensitivity, specificity, PPV and NPV of the ELISA-IgM and PCR assays for the determination of acute infection in patients suffering from CAP due to *C. pneumoniae, M. pneumoniae* and *L. pneumophila.*

Assay	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	95% CI*			
C. pneumoniae				
IgM	19 (11.84-28.07)	93 (86.11-97.14)	73.08 (54.42-86.0)	53.45 (50.73-56.15)
PCR	43 (33.14-53.29)	80 (70.82-87.33)	68.25 (57.77-77.17)	58.39 (53.56-63.07)
M. pneumonia				
IgM	6 (2.23-12.60)	97 (91.48-99.38)	66.67 (33.97-88.61)	50.79 (49.28-52.29)
PCR	0 (0.00-3.62)	100 (96.38-100.00)	NA	NA
L. pneumophila				
IgM	14 (7.87-22.37)	89 (81.17-94.38)	56 (37.79-72.72)	50.86 (48.24-53.47)
PCR	7 (2.86-13.89)	96 (90.07-98.90)	63.64 (34.59-85.28)	50.79 (49.12-52.47)

*Data percentage with 95% confidence interval. NA: not applicable (ignored values).

DISCUSSION

Overall, seroprevalence of the three atypical pathogens in participating patients was 64%; involved 26% *C. pneumoniae,* 8% *M. pneumoniae,* and 30% concurrent detection of two or more pathogens in CAP patients. The general prevalence of these agents in hospitalised CAP cases worldwide was lower than our findings (40%-60%) (3,4,15-17). Based on ELISA-IgG, the prevalence rate of *C. pneumoniae* (40%) was lower than than reported in adult patients from central Jordan and other countries (50%-70%) (11,18,19); but it was analogous to the recently published infection frequency in patients from Southern Jordan (6). Whilst the herein documented prevalence rate of *M. pneumoniae* (22%) in patients was in accordance with worldwide reported rates (3%-40%) (17,20-23), but higher than that stated for patients of central Jordan (12). Intriguingly,
L. pneumophila has been detected in 1%-16% of atypical pneumonia cases in different countries (17,22-24). Relatedly, reports from several countries demonstrated detection of anti-*M. pneumoniae* and anti-*L. pneumophila* IgG in healthy adults at rates (30%-86%) and (6-20%), respectively (6,8,11,16,25,26); frequencies that were relatively higher than our findings (Table 1). A significant 1.6 folds (p=0.02) and 4-folds (p<0.001) increase in the overall anti-*C. pneumoniae* and anti-*M. pneumoniae* IgG positivity in CAP cohort over asymptomatic controls indicated a possible association between these two pathogens and CAP in individuals of Southern Jordan.

Diagnosis of acute infections due to atypical agents is based on detecting a fourfold increase in IgG titer between acute-phase and convalescent serum, IgG titer ≥1:512 and/or IgM titer ≥1:16 in a single serum were adopted as serological criteria worldwide including population studies in Jordan (1,6,9,11,27-29). PCR is considered a second method to detect acute infections. Production of antibodies may delay in some infections and obtaining a paired serum requires 3-4 weeks (2); the level of IgM may not increase in response to re-infections in adults (29,30) and an acute increase in IgG titers in patients could be due to past exposure and not indicative of acute infection (31). These criteria are important in retrospective studies but clinically inconvenient when rapid detection of the etiological agent is compulsory and initiation of proper treatment protocols in a timely manner is demanding.

Assessed by ELISA-IgM as a tool for acute infection detection, the prevalence rate of *C. pneumoniae* was significantly 2.8 folds higher in CAP patients than controls (19% vs 7%, p= 0.01); whilst insignificant differences were noticed between detection rates of *M. pneumoniae* and *L. pneumophila* in CAP and control cohorts (6% and 14% versus 3% and 11%, respectively; Table 2). The frequency of *C. pneumoniae* detection reported herein was consonant with the epidemiological data from other countries which ranged 6%-22% (16,30,32); it was lower than previously reported results in uncontrolled and case-control studies in Jordan, which were 23% and 27.1%, respectively (6,9). Meanwhile, *M. pneumoniae* was detected in a frequency analogous with those documented earlier in several studies counting results from central and north Jordan (4%-21%) (10,12,16,22,24). Furthermore, the detection rate of anti-*L. pneumophila* IgM in CAP cases coincided with reports from India (11%-15%) (24,33) but was higher than formerly reported results in different epidemiological studies, including north Jordan (2%-9%) (2,10,23).

The detection rate of *C. pneumoniae* DNA in nasopharyngeal samples was significantly higher in CAP than in control participants (43% vs 20%, p< 0.001). Meanwhile, an insignificant difference in detection rate of *L. pneumophila* DNA was observed between patient and control cohorts (7% vs 4%, p= 0.3); *M. pneumoniae* was not detected in either CAP or control subjects by PCR (Table 2). Detection frequency of *C. pneumoniae* DNA by PCR in CAP adults is in agreement with recently published data from Southern Jordan (6) but higher than reported frequencies in respiratory tract infected patients worldwide (2%-23%) (15,17,34). Moreover, *L. pneumophila* DNA was detected in an analogous frequency to those reported in other studies (<1%-18%) (15,17,35); noteworthy, its nucleic acid was found in 53.8% of patients in uncontrolled study from Sudan using multiplex PCR (34).

The insignificant difference in detection frequency of anti-*M. pneumoniae* IgM between controls and CAP cases might be attributed either to an early sampling before production of the IgM in CAP patients as well as that the titer of IgM doesn't increase in adults in response to re-infections, or its low prevalence rate in populations from Southern Jordan. The absence of *M. pneumoniae* DNA in nasopharyngeal samples of seropositive CAP individuals might be due to weak efficiency of PCR in detecting *M. pneumoniae* DNA, an undetectable bacterial load, and pathogen shedding from respiratory tracts due to previous antibiotic treatments. Thus, the role of this bacterial agent in CAP occurrence was excluded during the time of this study, particularly as *M. pneumoniae* infection occurs in cyclic outbreaks every 3-7 years (5), its occurrence is rare in Jordanian CAP individuals and it is more common in children than adults (12,14); though, *M. pneumoniae* DNA has been identified in 1%-25% CAP cases worldwide (15,17,21,23,24,34). Moreover, absence of IgG response to *L. pneumophila* with insignificant detection rates of anti-*L. pneumophila* IgM and PCR in both CAP and control cohorts could be due to the insensitivity of used tests in detecting *L. pneumophila* infections, especially as asymptomatic carriage is not fully documented; cross reactivity in IgM detection test; or low prevalence of this pathogen in the studied population. Nevertheless, it was documented that continuous exposure of individuals to environmental sources contaminated with *L. pneumophila* might triggers immune response and causes elevation in Ig titers in 1%-30% of healthy individuals without clinical symptoms (2,36).

PCR assay was more sensitive in the diagnosis of *C. pneumoniae* acute infection than ELISA-IgM (43% vs 19%) but was less specific (80% vs 93%) with comparable diagnostic utility (PPV and NPV values). Conversely, ELISA-IgM was slightly more sensitive than PCR in detecting *M. pneumoniae* (6% vs 0%) and *L. pneumophila* (14% vs 7%) acute infections with comparable specificities but with lower accuracy in diagnostic utility than PCR in case of *L. pneumophila* (lower PPV value). Low sensitivity of IgM with a single serum may attributed to its delayed production in some infections and weak response at reinfections in adults. Therefore, if paired sera were obtained, IgM sensitivity in detecting acute infections might have been improved. Detection of bacterial DNA in serological negative patients was predicted either due to early sampling time before immune response was initiated or because of pathogen persistence. Whilst PCR negative results in serological positive cases could be due to past infections, an undetectable bacterial load, previous antibiotic treatments, pathogen shedding from respiratory tracts, presence of PCR inhibitors, or assay technical problems associated with unsuccessful sample processing.

Overall, concurrent infections were detected in 30% of all CAP patients by serology; *C. pneumoniae* and *L. pneumophila* were detected in 14 patients (14%), *C. pneumoniae* and *M. pneumoniae* or *M. pneumoniae* and *L. pneumophila* were identified in six patients (6%) and the three pathogens were found in four CAP individuals (4%). It was possible to identify the DNA of both *C. pneumoniae* and *L. pneumophila* in seven patients from the entire CAP cohort (7%); all those that were PCR positive to *L. pneumophila* demonstrated positivity to *P. pneumoniae*. The presence of one pathogen might prime the coinfection by the other pathogen; that explains the reported 25%-48% concurrent infections in CAP cases by different studies (37,38).

Study limitations include reliance on a single serum without following it with a paired serum (seroconversion), incapability of determining the endpoint titer of antibodies against each atypical pathogen and using nasopharyngeal samples for pathogens' DNA detection instead of sputum might influence the sensitivity of PCR. The study depended on blood samples for detection of *L. pneumophila* without incorporating it with urine antigen test, and there is no agreed evaluation on diagnostic accuracy of ELISA. Obtaining second convalescent sera from patients during hospital stay was impractical and challenging; and delay in seroconversion results may not be useful in acute infection diagnosis or during outbreaks. Nevertheless, serodiagnosis of acute infection based on a single serum sample seems to be more realistic in clinic facilities as the rapid decision for empirical treatment of an infection is based often on a single serum sample.

Based on the current findings, *C. pneumoniae* may play a causal role in CAP infection in population of Sothern Jordan; absence of bacterial DNA in respiratory samples from some of the *C. pneumoniae* seropositive CAP patients indicated past infections. Both *M. pneumoniae* and *L. pneumophila* have low prevalence rates in studied population. The strong correlation between PCR assay and IgM in detecting acute infections points out that PCR could improve the clinical utility of serological methods, with caution in single samples. Simultaneous use of ELISA and PCR in clinical facilities might allow rapidity and improvement in diagnosis of acute respiratory infections and precision in identifying the etiological pathogen that may play role in establishing CAP infections when convalescent samples are unattainable.

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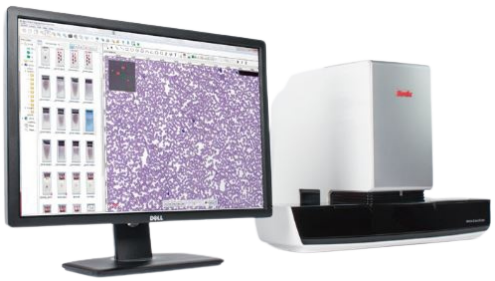

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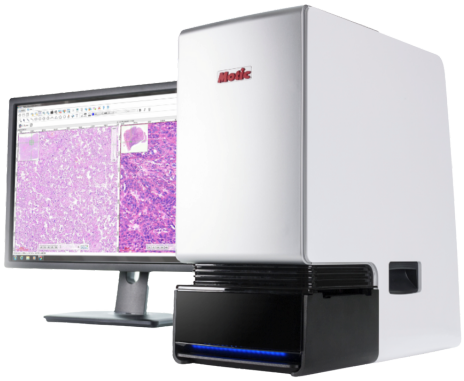

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ORIGINAL ARTICLE

Influence of storage time on stability of routine coagulation parameters (international normalised ratio, activated partial thromboplastin time and fibrinogen) at room temperature

Richard M Chen, Yii Sen Wee and Rhonda Lucas

ABSTRACT

Objectives: Extending the maximum acceptable specimen age for testing/retesting some routine coagulation parameters has many benefits (e.g., reagent evaluation, fibrinogen add-on for disseminated intravascular coagulation, or addressing sample delay during snow in Otago/Southland). This study assessed such possibility, where stability of international normalised ratio, activated partial thromboplastin time and Fibrinogen results were examined in relation to storage time.

Methods: From each participating individual (50 total), four citrate tubes were collected. A baseline tube was centrifuged and tested for international normalised ratio, activated partial thromboplastin time and fibrinogen at time of arrival. The other three tubes were kept as whole blood. After 24, 48, and 72 hours respectively from time of collection, one tube was taken for centrifuging and tested for international normalised ratio, activated partial thromboplastin time, and fibrinogen. In addition, centrifuged tubes were retested for international normalised ratio and fibrinogen after 24, 48, and 72 hours respectively (centrifuged fibrinogen after 96 hours) from time of collection. All specimens were kept at room temperature.

Results: The mean bias of international normalised ratio, activated partial thromboplastin time, and fibrinogen at after 24h, 48h, and 72h (centrifuged fibrinogen after 96h) were calculated and compared with specific allowable limits of performance. For both centrifuged and uncentrifuged specimens, international normalised ratio variations up to 72h all passed Royal College of Pathologists of Australasia allowable limits of performance (±0.3); fibrinogen variations up to 72h (centrifuged fibrinogen up to 96h) all passed European Federation of Clinical Chemistry and Laboratory Medicine allowable limits of performance (±10.7%). Activated partial thromboplastin time had a clear increasing trend, and all of its variations failed the European Federation of Clinical Chemistry and Laboratory Medicine allowable limits of performance (±2.7%).

Conclusion: Evidence supports extension of maximum acceptable age of uncentrifuged specimens for international normalised ratio and fibrinogen tests to 72h. Changing the maximum allowable age of activated partial thromboplastin time is not recommended. For centrifuged specimens, we can extend maximum acceptable age for international normalised ratio to 72h and fibrinogen to 96h.

Key words: International normalised ratio (INR), activated partial thromboplastin time (APTT), Fibrinogen, specimen age, result stability.

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INTRODUCTION

Routine coagulation tests that are done the most in the haematology department of a medical laboratory include PT (prothrombin time)/INR (International normalised ratio), APTT (activated partial thromboplastin time), and fibrinogen. PT/INR measures the ability to clot by extrinsic pathway, which depends on activities of Factor VII and II, V, X, and fibrinogen. INR is the normalised ratio of patients' PT in relation to a mean normal PT, which is standardised depends on the types of tissue factor used by the reagents. Many community patients who are on warfarin, (a vitamin K inhibitory anticoagulant), need to have their INR level measured and monitored regularly for therapeutic reasons. APTT measures the ability to clot by intrinsic pathway and it mainly depends on activities of Factor VIII, IX, XI, XII, as well as Factor II, V, X, and fibrinogen. Fibrinogen (also known as Factor I) is essential in the process of haemostasis for its role in converting to fibrin by enzymatic reaction with thrombin to form the stable fibrin-based blood clot.

In order to maintain high laboratory diagnostic quality, routine coagulation tests require pre-analytical elements of sodium citrated specimens to be strictly controlled before testing. These include storage time, temperature, and the status of the specimens (kept as whole blood or centrifuged to obtain separated platelet poor plasma).

The Clinical and Laboratory Standard Institute (CLSI) guideline requires testing of INR, APTT, and fibrinogen in 0.109M sodium citrate samples (3.2% buffered) (1). The current acceptable age also set by the guideline allows specimens to be kept as whole blood for INR at maximum 24 hours; for APTT at maximum 4 hours (if it is for unfractionated heparin analysis, maximum age is one hour); for fibrinogen at maximum 4 hours (1). Based on this guideline, the maximum acceptable ages for INR, APTT, and fibrinogen tests as either whole blood or centrifuged samples at room temperature in Southern Community Laboratories (SCL) Dunedin Haematology Department are set and listed in Table 1.

The short time windows for these parameters pose some challenges on transportation and storage of the specimens. SCL Dunedin regularly receives out-of-town citrate tubes for INR monitoring. Located in Otago/Southland, delay of sample arrival is common in the event of snow or icy road conditions. In this situation, patients may need to re-bleed, which causes extra time and financial cost. Hence, the original aim of this study was to examine whether we can extend the maximal acceptable time frame of INR testing. Testing for fibrinogen and APTT were included later into the study, adding up more context on examining the extension of routine coagulation sample age.

Halfway during the study, we added two more test groups. Being the largest clinical medical laboratories in Otago/ Southland, SCL Dunedin often receives requests from rural laboratories of sending 'warfarinised' citrate tubes to them for their reagent evaluation due to their lack of patients. However, the laboratory can only send specimens away after the requested tests are done, resulting in them all being centrifuged. Therefore, we evaluated the stability of INR from spun citrate tubes over time. In addition, we also examined the stability of fibrinogen from centrifuged citrate tubes.

For both studies on INR stability from specimens stored as whole blood and centrifuged tubes, we also checked the differences of performance from 'warfarinised' and non-'warfarinised' patients. This is to determine whether presence of warfarin would affect INR stability.

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METHODS

A total of 50 subjects from Dunedin community participated in this study at SCL Filleul Street Collection Centre in the period from 15/3/21 to 4/5/21. We obtained the consent to take blood from all participating subjects by letting them sign consent stickers, which were attached on their laboratory request forms.

In this study, each participating subject was invited to provide four 0.109M sodium citrate samples (3.2% buffered). A baseline tube was centrifuged and tested for INR, APTT, and fibrinogen at time of arrival at the laboratory. The other three tubes were kept as whole blood. After 24, 48, and 72 hours respectively from time of collection, one tube not centrifuged was taken for centrifuging and tested for INR, APTT, and fibrinogen. By doing so, we obtained the variations of INR, APTT, and fibrinogen from unspun tubes of each subject over 72 hours.

Stability of whole blood INR was measured for all participants (50 total). Among those, 24 subjects were on warfarin, one on dabigatran, and 25 subjects not on any anticoagulation. 34 subjects had their whole blood APTT variation measured, and 39 subjects had their whole blood fibrinogen variation measured. Since APTT and fibrinogen tests were not included in this study initially, the amounts of subjects tested were less than total amount of participants.

Test tubes for stability of centrifuged INR were obtained from 30 accessioned patient specimens requested for real routine coagulation tested at the laboratory, after the tests were completed. This was approved by Yii Sen Wee, the Head of Haematology Department and Rhonda Lucas, the Coagulation Specialist. Among these specimens, 20 patients were on warfarin and 10 were not. All tubes were reused and tested for INR after 24, 48, and 72 hours respectively from their collection time.

Fibrinogen stability from centrifuged tubes was evaluated by re-using the baseline tubes from those 50 test subjects. In total, 29 tubes were selected based on their haematocrit (to provide an assessment of how much plasma would be available). After 24, 48, and 96 hours respectively from collection time, fibrinogen from these tubes was measured.

Storage temperature was maintained at the room temperature (circa 21°C) of SCL Dunedin for all samples throughout the period of the study. All tests were performed on either one of the two Sysmex CS2500 automated coagulation analysers in SCL Dunedin Haematology, depending on which one was rostered for routine coagulation testing. Reagents used include Dade Innovin™ for INR testing; for APTT there are Actin FS™ and 0.025mol/L calcium chloride solution; for fibrinogen there are Siemens Thrombin and Owren's Veronal Buffer. Both analysers performed QC daily and maintained good diagnostic capability.

RESULTS

The individual value variation from each test groups was illustrated as line charts. For all five test groups, with the help of software Analyse-it in Excel, the mean and median, and Bland-Altman of INR, APTT, or fibrinogen at baseline, after 24, 48, and 72 hours respectively were plotted as graphs. The mean bias for each time points were calculated and listed in Table 2.

All the raw data are presented in the Supplementary Table available online from the NZIMLS website.

Whole blood INR

Figure 1 showed the INR variations of each individual over 72 hours. Figure 2 provides the mean and median of INR at different time points. Both figures separated the subjects into 'warfarinised' and non-'warfarinised'. The changes in INR over time for both groups showed similar stable patterns. Therefore, presence of warfarin was not observed to cause significant INR variation in whole blood. We then combined warfarin and non-warfarin groups together for whole blood INR interpretation. The combined mean and median plot for whole blood INR was also shown in Figure 2.

Figure 3 provides three Bland-Altman plots. From left to right, each plot showed the whole blood INR differences of all individuals between baseline and after 24, 48, and 72 hours respectively. The mean bias was calculated for each storage time: 0.02 (24 hours), 0.05 (48 hours), and 0.12 (72 hours). They were assessed against the RCPA Analytical performance specification of INR, which is set at ±0.3 (2). All mean differences passed the RCPA guideline, indicating that INR is stable up to 72 hours when stored as whole blood at room temperature.

Whole blood APTT

Figure 4 included a line chart of the APTT variation of each individual over 72 hours and a mean and median APTT plot at different time points. An increasing trend was observed from the line chart. The mean and median APTT also increased significantly (mean over 12 seconds; median over 9 seconds). The Bland-Altman plots (Figure 5) for APTT between baseline and after 24, 48, and 72 hours respectively also showed significant increase in APTT results over time. The mean bias for each time point was shown in percentage for comparison with EFLM (European Federation of Clinical Chemistry and Laboratory Medicine) desirable within-individual biological variation of APTT, which is 2.7% (3, 4). The results were 13.87% (24 hours), 26.62% (48 hours), and 34.77% (72 hours). Clearly, none of the mean bias result passed the EFLM guideline, which means APTT is not stable from 24 hours as whole blood at room temperature.

Whole blood fibrinogen

Figure 6 included a line chart of fibrinogen variation of each individual over 72 hours and a mean and median fibrinogen plot at different time points. Subjects showed generally flat lines from the line chart. The mean and median fibrinogen showed little variation (maximum increase of 0.1 from median baseline result). Figure 7 demonstrates the Bland-Altman plots for

fibrinogen within-individual variations between baseline and after 24, 48, and 72 hours respectively. for each time point, the mean bias was shown in percentage for comparison with EFLM desirable within-individual biological variation of fibrinogen, which is 10.7% (3, 4). The mean bias values were -0.50% (24 hours), -0.03% (48 hours), and 0.80% (72 hours). All the mean bias results passed the EFLM guideline, which means fibrinogen is stable up to 72 hours as whole blood at room temperature.

Centrifuged INR

Figure 8 showed the INR variations of each individual over 72 hours. Figure 9 provides the mean and median of INR at different time points. Both figures separated the subjects into 'warfarinised' and non-'warfarinised'. The changes in INR over time for both groups showed similar stable patterns. Therefore, presence of warfarin was not observed to cause significant INR variation in reused centrifuged tubes. We then combined warfarin and non-warfarin groups together for spun INR interpretation. The combined mean and median plot for whole blood INR was also shown in Figure 9.

Figure 10 gave three Bland-Altman plots. From left to right, each plot demonstrated the whole blood INR differences of all individuals between baseline and after 24, 48, and 72 hours respectively. The mean bias was calculated for each time point: -0.03 (24 hours), 0.03 (48 hours), and 0.09 (72 hours). These values were assessed against the RCPA analytical performance specification, which is the same ±0.3 as mentioned above. All mean bias results passed the RCPA guideline, indicating that for centrifuged citrate tubes at room temperature, INR is stable up to 72 hours.

Centrifuged fibrinogen

Figure 11 included a line chart of fibrinogen variation of each individual over 96 hours and a mean and median fibrinogen plot at different time points. Subjects showed generally flat lines from the line chart. The mean and median fibrinogen showed little variation (maximum increase of 0.1 from median baseline result). Figure 12 demonstrates the Bland-Altman plots for fibrinogen within-individual variations between baseline and after 24, 48, and 96 hours respectively. The mean bias for each time point was shown as a percentage for comparison with EFLM fibrinogen allowed limit of performance, which is 10.7 as mentioned above. Overall, the mean bias values were 1.85% (24 hours), -0.64% (48 hours), and 0.48% (96 hours) respectively. All the mean differences passed the EFLM guideline, which means fibrinogen is stable up to 96 hours in centrifuged citrate tubes at room temperature.

Table 1. The current standard for acceptability age of coagulation samples in SCL Dunedin Haematology Department.

Test	Whole blood (room temperature)	Centrifuged (room temperature)
PT/INR (On Warfarin)	<24h	<24h
PT/INR (Not on Warfarin)	<8h	<8h
APTT (On Heparin)	<1h	<2h
APTT (For Special Coagulation)	<4h	<4h
APTT (For Routine Coagulation)	<8h	<8h
Fibrinogen	<24h	<24h

Table 2. Calculated mean bias of all five test groups done in the study, along with indications of pass/fail against allowed limits of performance.

	Calculated mean bias	Baseline vs. after 24h	Baseline vs. after 48h	Baseline vs. after 72h	Baseline vs. after 96h	Allowed limit of performance (ALP), guideline used	Test group passed/failed ALP
INR (Whole blood)	In absolute number	0.02	0.05	0.12	/	RCPA (6) ±0.3	PASSED
APTT (Whole blood)	In %	13.87%	26.62%	34.77%	/	EFLM (2,3) ±2.7%	FAILED
Fibrinogen (Whole blood)	In %	-0.50%	-0.03%	0.80%	/	EFLM (2,3) ±10.7%	PASSED
INR (Centrifuged)	In absolute number	-0.03	0.03	0.09	/	RCPA (6) ±0.3	PASSED
Fibrinogen (Centrifuged)	In %	1.85%	-0.64%	/	0.48%	EFLM (2,3) ±10.7%	PASSED

Table 3: Comparison of mean within-subject % variation in PT/INR from previous studies and our study.

Test	Number of test subjects	Mean % change from baseline after 24h	Mean % change from baseline after 48h	Mean % change from baseline after 72h
Our study (whole blood INR)	50	1.13	3.08	6.45
Totzke *et al* (PT whole blood) (11)	14	-5.4	-12.2	-12.1
Zürcher *et al* (PT whole blood) (12)	59	-4.2	-10.8	NA
Our study (centrifuged INR)	30	-1.23	1.88	4.30
Heil *et al* (PT plasma) (13)	40	0 to -10%	0 to -10% (healthy) -10% to -20% (heparin)	-10% to -20%
Linskens *et al* (PT plasma) (14)	20	-3.9	-0.1	NA

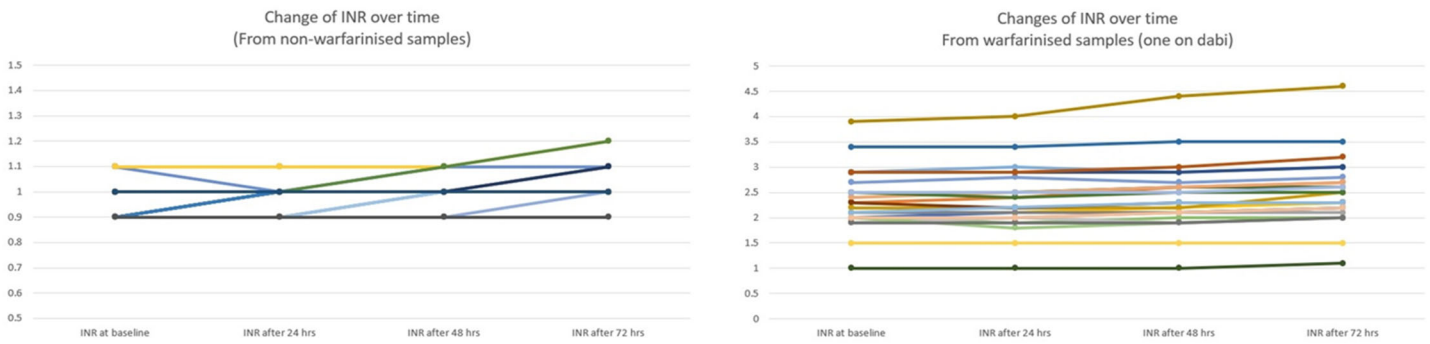

Figure 1. Line Charts of whole blood INR variation over time from non-warfarinised (left) and warfarinised (right) samples. Note one result from a patient on dabigatran is included in the chart on the right (The bottom line). However, its trend showed no significant difference from other lines.

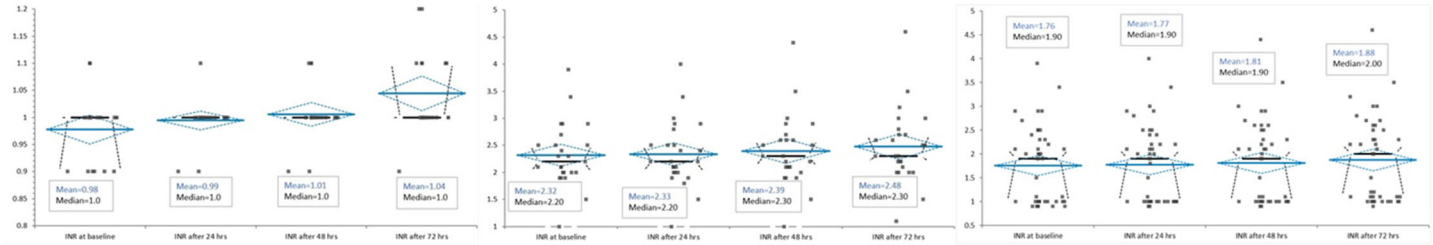

Figure 2. Mean and Median plots of whole blood INR over time from non-warfarinised (left), warfarinised (middle), and combined (right) samples.

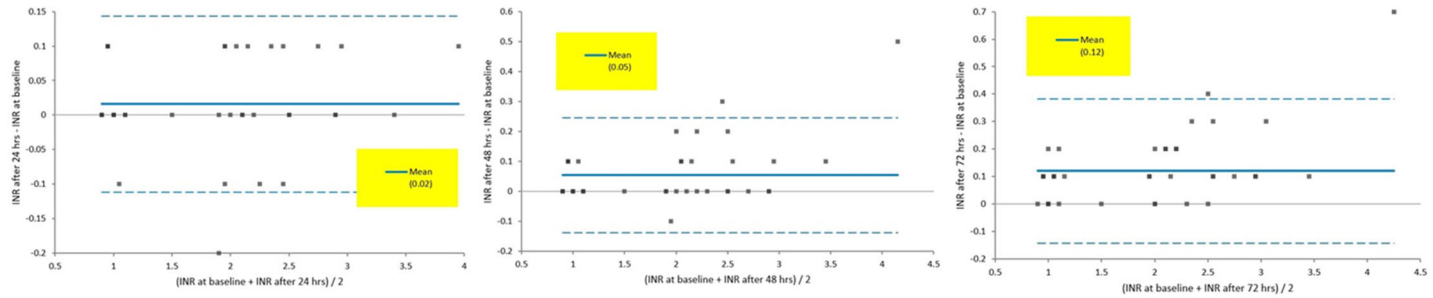

Figure 3. Bland-Altman plots of whole blood INR variation for all individuals between baseline and after 24h (left), 48h (middle), and 72h (right).

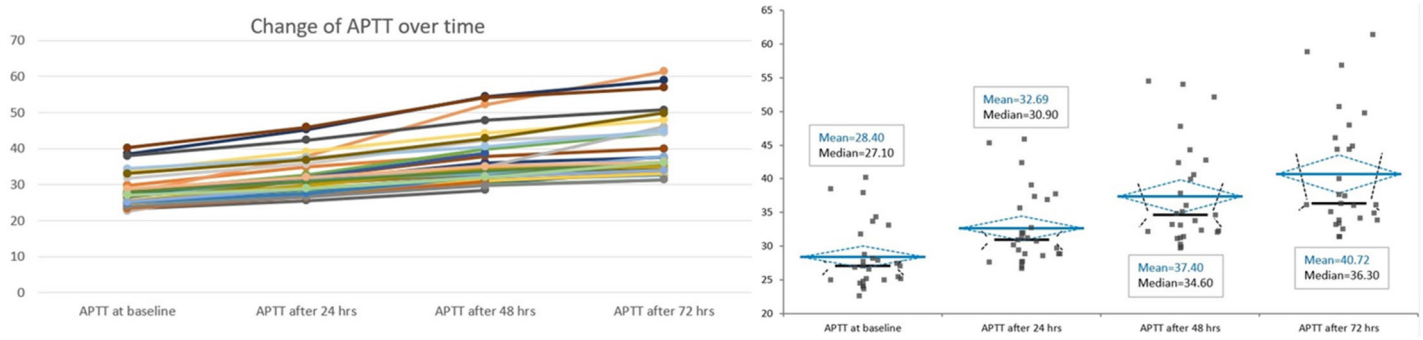

Figure 4. Line chart of whole blood APTT variation over time (left) and Mean and median plot of whole blood APTT variation over time (right).

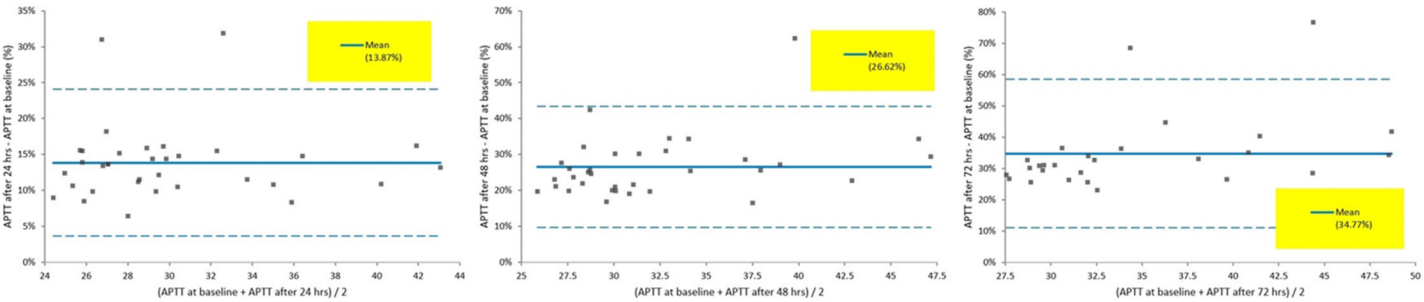

Figure 5. Bland-Altman plots of whole blood APTT variation for all individuals between baseline and after 24h (left), 48h (middle), and 72h (right).

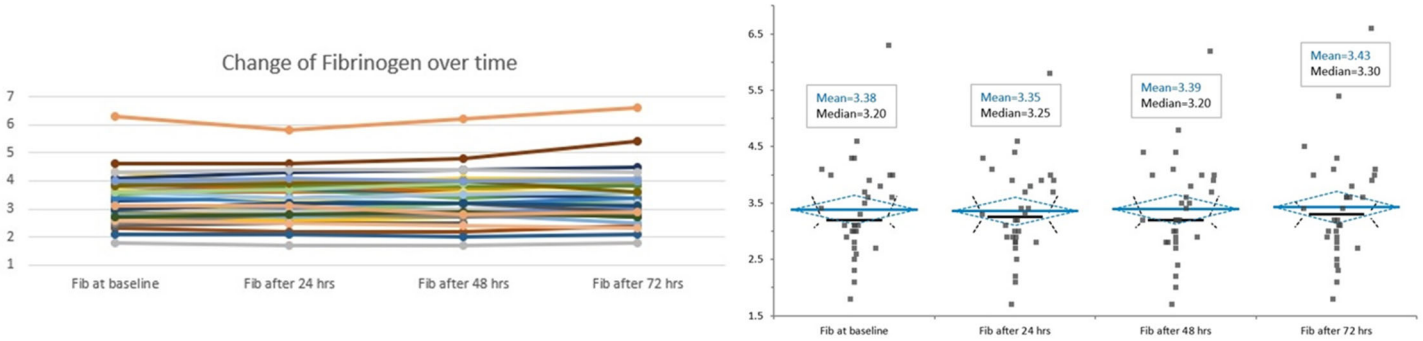

Figure 6. Line chart of whole blood fibrinogen variation over time (left) and Mean and median plot of whole blood fibrinogen variation over time (right).

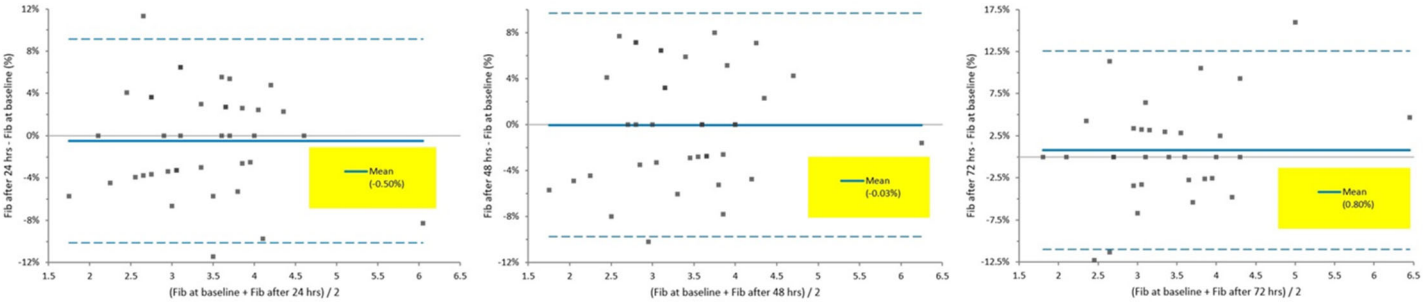

Figure 7. Bland-Altman plots of whole blood fibrinogen variation for all individuals between baseline and after 24h (left), 48h (middle), and 72h (right).

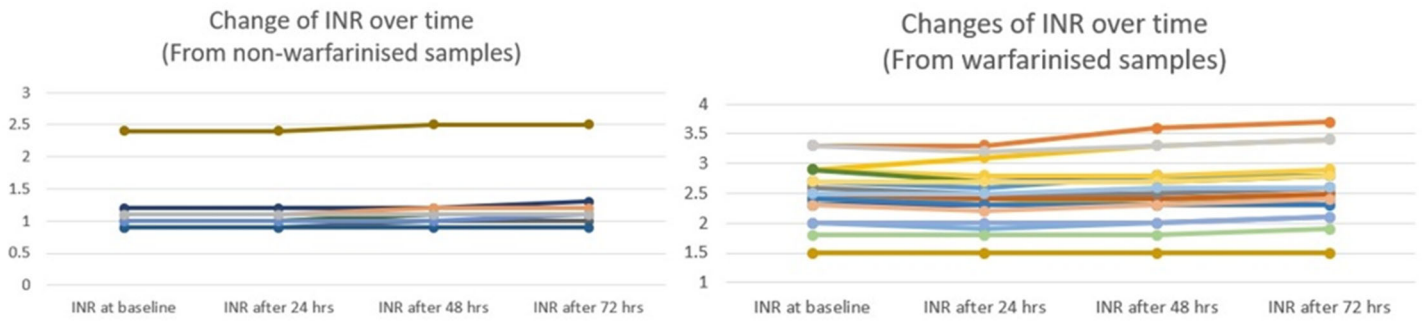

Figure 8. Line Charts of centrifuged INR variation over time from non-warfarinised (left) and warfarinised (right) samples. (The isolated top line on the left belongs to a patient with liver cirrhosis.)

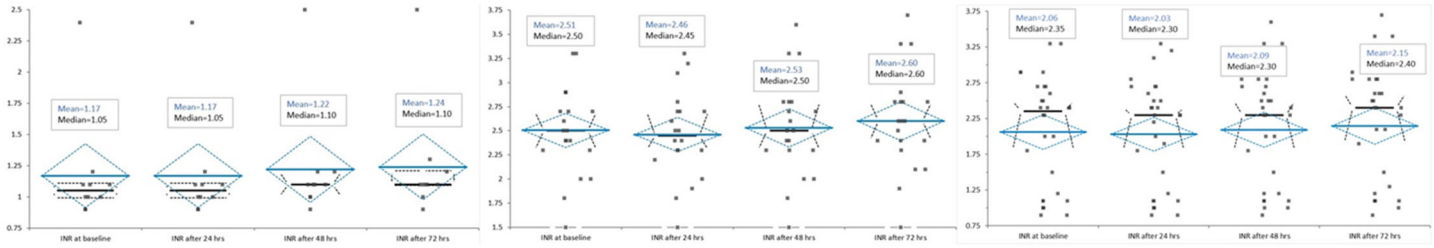

Figure 9. Mean and Median plots of centrifuged INR over time from non-warfarinised (left), warfarinised (middle), and combined (right) samples.

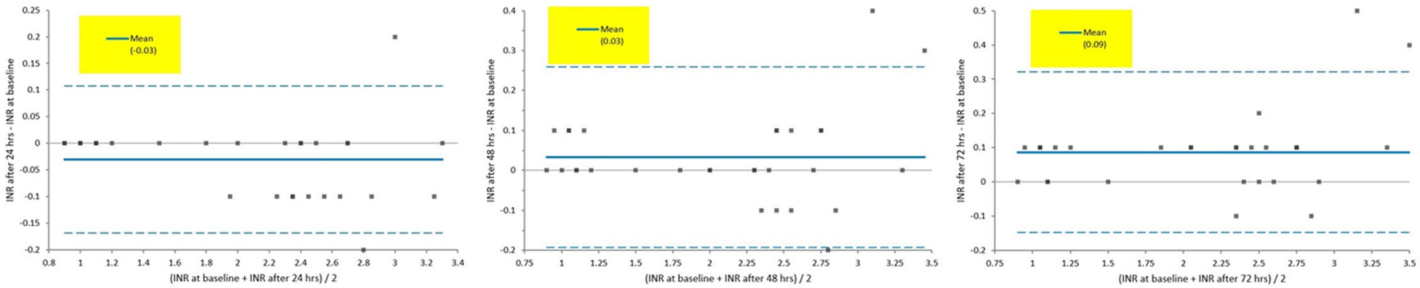

Figure 10. Bland-Altman plots of centrifuged INR variation for all individuals between baseline and after 24h (left), 48h (middle), and 72h (right).

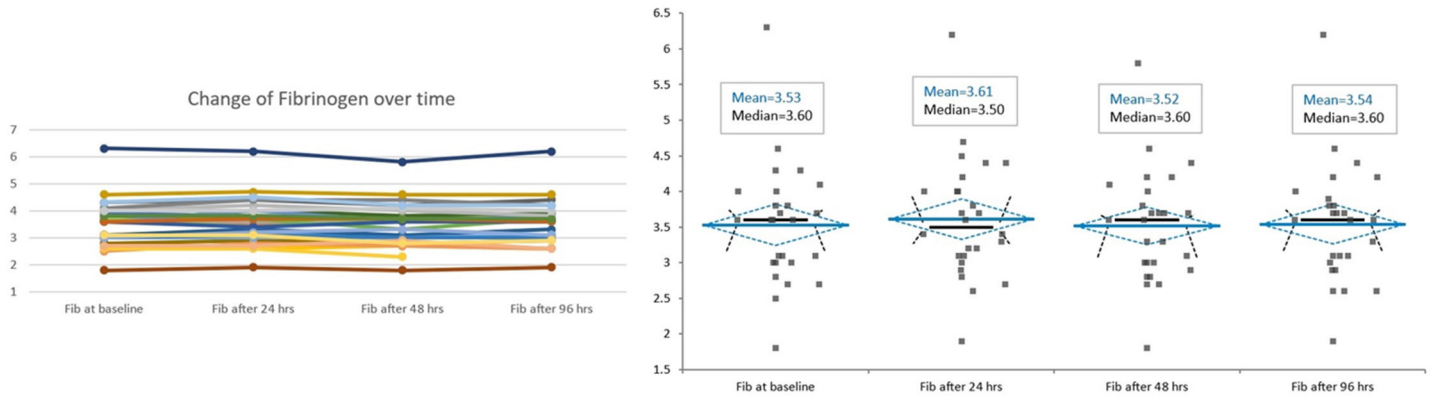

Figure 11. Line chart of centrifuged fibrinogen variation over time (left) and Mean and median plot of spun fibrinogen variation over time (right).

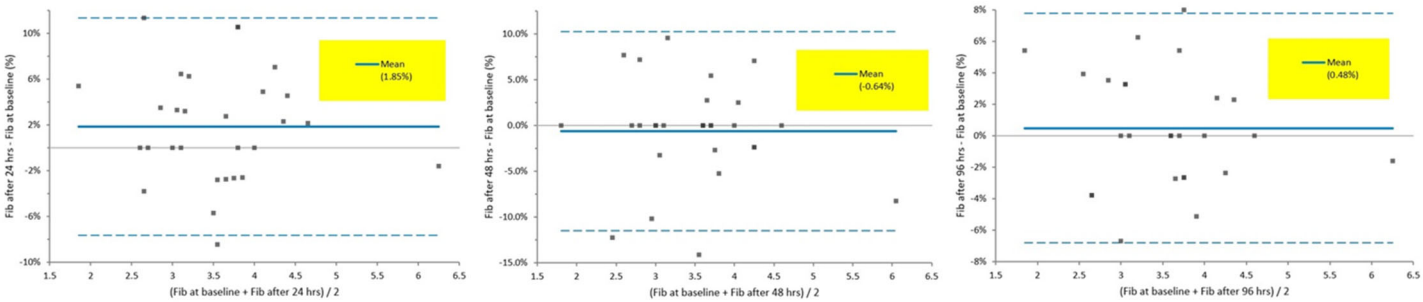

Figure 12. Bland-Altman plots of centrifuged fibrinogen variation for all individuals between baseline and after 24h (left), 48h (middle), and 96h (right).

DISCUSSION

Many previous studies undertaken on INR/PT stability only tested specimens over a storage time of maximum 24 hours, for both samples stored as whole blood (5,6), or separated into plasma (centrifuged) (7-10). Challenges to find supporting studies on INR stability over 72 hours at room temperature did exist. Of the four publications that include INR/PT stability testing over 24h were found, two were based on centrifuged plasma and the other two were based on whole blood. Table 2 summarises the performances of the previous studies and compared them with the current study. Overall, our INR variation over time showed slight increase but was not clinically significant according to the RCPA standard.

From whole blood

Totzke *et al.* (11) showed PT results from whole blood tubes stored at room temperature had significant decrease at 5.4% at 24 hours, 12.2% at 48 hours, and 12.1% at 72 hours following collection. However, this study had smaller number of samples (n=14). Zürcher e*t al.* (12) stated change in PT at 24 hours was less than 10% and less than 15% at 48 hours. Our results showed similar variation patterns as the two studies listed.

From centrifuged tubes

Heil *et al.* (13) stated the stored plasma at room temperature is stable (variation less than 10%) for up to 48 hours among healthy subjects and 24 hours among heparinised patients. Furthermore, Linskens et al (14) stated PT in plasma stored at room temperature is stable up to 48 hours. Our results were more stable than the two studies listed.

The presence of warfarin did not affect performances of INR testing on either on-warfarin or not-on-warfarin patient groups (whole blood: Figure 1,2; Centrifuged: Figure 8,9). A supporting study by Baglin *et al.* demonstrated that INR was stable for 'warfarinised' patients up to three days and Innovin™ (the thromboplastin reagent we used in the current study) gave the most stable INRs (15). It further proved that within 72 hours of storage at room temperature, testing on 'warfarinised' samples would not cause clinically significant change from baseline results. There was indeed one warfarinised individual in our study (Figure 1, the top line on the chart to the right) whose INR result varied from 3.9 to 4.6 over 72 hours. Due to sample size, we might not rule out a different stability pattern of INR for over-anticoagulated patients (INR>3.0), but it would not cause critical concern by our lab protocol (INR≤5.0). A subject on dabigatran was also included in the study (Figure 1, the bottom line on the chart to the right) but the INR variation was not clinically significant (from 1 to 1.1 over 72 hours).

Whole blood APTT were expected to be significantly elevated over 72 hours. Based on previous studies it is known Factor V and VIII activity will decline significantly after 6-8 hours (5, 12). Statistically significant change of APTT measurements were observed when sample age was >24 hours in the study undertaken by Zürcher *et al.* (12). Totzke *et al.* (11) also showed significant APTT increase over 72 hours. We had proven that samples requested for APTT are not stable after 24 hours and onward. We did not perform APTT on samples stored as whole blood at room temperature within sample age of 24 hours (e.g., test APTT at baseline, after 4, 8, 12, 16 hours, etc., respectively) because we would not want to increase workload for our nightshift lab staff. However, such practice is recommended for future studies as an improvement. Continue to follow the current criteria of APTT maximum allowable age (eight hour maximum) is recommended.

In our study, fibrinogen level has been proven to be stable up to 72 hours in whole blood and up to 96 hours in centrifuged tubes. Studies have shown that fibrinogen level is stable up to seven days when stored at room temperature (13,16), which correlated with our findings. This also justified the action to extend centrifuged fibrinogen measurement to 96 hours and omit measurement at 72 hours, allowing the laboratory to perform fibrinogen add-on from centrifuged citrate tubes over a time course of five days. No significant trend of fibrinogen variation over time has been observed in this study. Some occasional negative mean bias (Table 2) might be accounted by a slow declining of fibrinogen activity over time or the Owren's Veronal Buffer we used was close to its maximum acceptable age, but neither of these two factors caused clinically significant change in fibrinogen measurement.

Overall, some potential interfering pre-analytical factors include one slight lipemic subject, two slight haemolysed subjects, and some specimens were not tested at the exact scheduled time point (maximum one-hour delay). Some participating subjects only had three tubes bled, therefore, evaluation of INR/APTT/fibrinogen after 72h could not be undertaken. However, these limitations did not affect the overall results significantly.

CONCLUSION

Based on our study, we are confident to extend the maximum allowable age of uncentrifuged specimens for INR and fibrinogen tests to 72 hours. Age requirement for uncentrifuged APTT should not be changed. For centrifuged specimens, maximum acceptable age for INR up to 72 hours and fibrinogen up to 96 hours can be extended.

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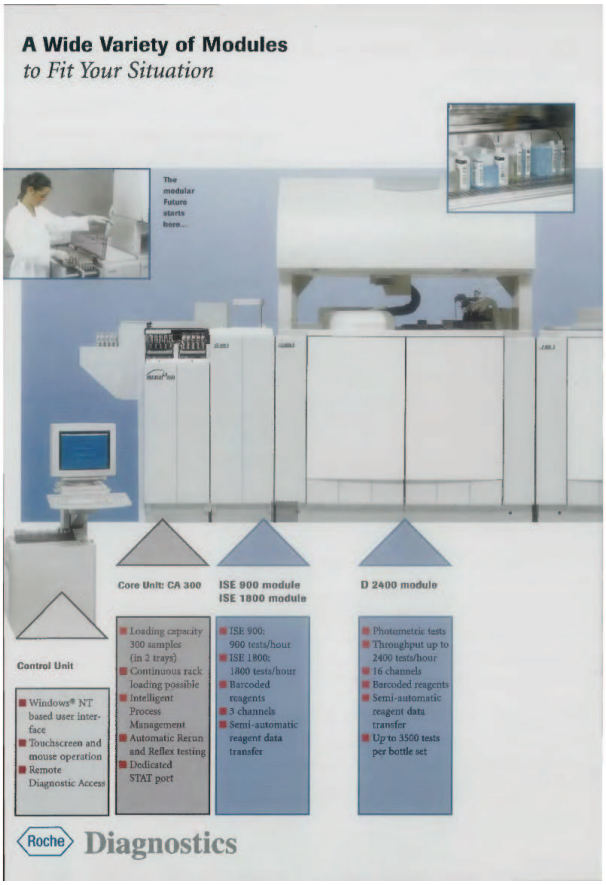

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ORIGINAL ARTICLE

Selected inflammatory and haemolytic indicators among adolescents living with sickle cell anaemia in a malaria-endemic population

Euphoria C Akwiwu, Josephine O Akpotuzor, Dorathy C Okpokam, Eme E Onukak, Stanley O Anyanwu and Valerie E Ugochi

ABSTRACT

Objectives: Transition between paediatric and adult care represents a critical period in the management of sickle cell anaemia. Both the sickling condition itself and malaria are associated with inflammation, thus, the need to investigate the impact of malaria infection on neutrophilic response, glutathione and bilirubin levels among adolescents with sickle cell anaemia in a malaria-endemic population.

Methods: This study was carried out among 68 steady-state sickle cell anaemia adolescent attending clinic at University of Calabar Teaching Hospital, Calabar-Nigeria. The subjects were asymptomatic for malaria. All tests were carried out by standard methods. Statistical analysis of data was carried out using SPSS 22.0. A p-value of ≤ 0.05 was considered to infer a statistically significant difference.

Results: Leucocyte counts were significantly higher, while neutrophil function rate was lower in sickle cell anaemia subjects compared to control subjects. Bilirubin mean values were also significantly higher while glutathione mean values were lower among subjects living with sickle cell anaemia. These derangements were heightened by malaria infection. Glutathione correlated negatively with total white cell count, neutrophil count and unconjugated bilirubin while a positive relationship was observed between the former and neutrophil function rate.

Conclusion: Asymptomatic malaria infection impacts negatively on immune response among persons living with sickle cell anaemia. This reveals an important intervention target for the transition from paediatric to adult care in the management of sickle cell anaemia in malaria-endemic areas.

Keywords: Sickle cell anaemia, inflammation, leucocytes, haemolysis

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INTRODUCTION

Sickle cell disease is a group of inherited disorders with significant contributions to childhood mortality, especially among persons of African, Middle East and Mediterranean nativity (1,2). Sickle cell anaemia (haemoglobin SS) is the disease variant predominantly found in Nigeria where its persistence is largely driven by inadequate healthcare coverage particularly in rural and sub-urban populations (3). This has been attributed to lack of premarital testing for haemoglobin types among prospective couples which in turn increases the probability of having children with sickle cell anaemia. While access to adequate healthcare greatly improves survival of affected individuals from childhood into adult life, the transition between paediatric care and adult care has been recognized as a critical period in the management of the disorder even in developed countries with improved healthcare (4-6). Thus, life expectancy among sickle cell anaemia patients continues to be a critical area of challenge in the management of the condition particularly among adolescents.

In addition to the known anaemia, platelet involvement and occlusive processes associated with sickling of red blood cells, there also exists overt inflammation alongside chronic haemolysis in sickle cell anaemia (1,7). Inflammation from bacterial infection has been mainly addressed through routine vaccination. Unfortunately, other infections such as that from *Plasmodium falciparum* which causes malaria has no vaccine yet and is endemic in Nigeria where children are particularly vulnerable (8-10). Certain factors have been identified as triggers for the onset of sickle cell-related crisis. These include malaria, exposure to excess cold or heat, physical and emotional stress, dehydration, fever and high altitude. Malaria-triggered crisis is common among sickle cell anaemia subjects in Nigeria (11-13). Sickle cell anaemia and malaria infection are associated with inflammation and independently induce phagocytic response and haemolysis (14-17). Thus, the Nigerian population of children with sickle cell anaemia could be at increased risk of inflammation. The main objective of this study was to investigate the impact of malaria infection on neutrophil count as well as its function rate, glutathione and bilirubin levels among adolescents with sickle cell anaemia in a malaria-endemic population.

METHODS

This study was carried out among 68 steady-state sickle cell anaemia adolescent attending clinic at University of Calabar Teaching Hospital, Calabar-Nigeria. The study subjects included equal numbers of male and female sickle cell anaemia patients with age and gender-matched Hb AA controls. 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 and the respective guardian. The subjects were asymptomatic for malaria. Malaria infection was determined by microscopy on direct thick and thin film. The leucocyte counts were carried out by automation using SMART-1 (three-part differentiation) Haematology Analyzer from Kinghawk Technology Co., Ltd, China. This analyser was controlled and calibrated according to manufacturer's instructions to ensure it was fit for use.

Neutrophil function rate was determined using the *Escherichia coli* culture method. In this method, *E coli* was sub-cultured in peptone water from a stock culture isolated in a nutrient agar medium. After incubating blood sample in the *E coli* broth, blood film was made and stained with Leishman stain prior to microscopic examination. Function rate was derived from the number of phagocytized neutrophils per the total number of neutrophils present.

Quantitative determination of glutathione (GSH) was carried out by enzyme-linked immunosorbent assay Method using kits from Bioassay Technology Laboratory, China. Total and conjugated bilirubin were determined using kits from Randox Laboratories Limited, UK.

Unconjugated bilirubin was derived mathematically from Total and conjugated bilirubin values. Statistical analysis of data (Student t-test and Pearson's correlation) was done using SPSS 22.0. A p-value of ≤ 0.05 was considered to infer a statistically significant difference.

RESULTS

Leucocyte counts were significantly higher, while neutrophil function rate was lower in sickle cell anaemia subjects compared to control subjects. Bilirubin mean values were also significantly higher while glutathione mean value was lower among subjects living with sickle cell anaemia as shown in Table 1.

Apart from absolute lymphocyte count, which showed no significant difference, other leucocyte counts were higher in asymptomatic malaria infection. Neutrophil function rate was however further reduced in this group of sickle cell anaemia subjects. There also occurred much higher levels of bilirubin values but lower glutathione level in the same group (Table 2).

Pearson's correlations shown in Table 3 indicate that glutathione correlated negatively with total white cell count, neutrophil count and unconjugated bilirubin while a positive relationship was observed between the former and neutrophil function rate.

Table 1. Selected indicators of inflammation and haemolysis among adolescents living with sickle cell anaemia .

Parameters	Hb AA Subjects (n=68)	Hb SS Subjects (n=68)	p-value
WBC ($\times 10^9$/l)	5.24 ± 1.26	11.44 ± 2.53	0.001
LYMPH ($\times 10^9$/l)	2.59 ± 0.57	4.28 ± 1.28	0.001
MXD ($\times 10^9$/l)	0.40 ± 0.18	1.42 ± 0.32	0.001
NEUT ($\times 10^9$/l)	2.24 ± 1.09	5.73 ± 2.32	0.001
NFR (%)	36.01 ± 4.44	25.84 ± 4.38	0.001
TB (µmol/l)	16.52 ± 2.68	34.36 ± 7.63	0.001
CB (µmol/l)	8.27 ± 1.64	15.19± 4.94	0.001
UB (µmol/l)	8.25 ± 1.99	19.17± 4.74	0.001
GSH (µg/l)	3.99 ± 1.88	1.45 ± 0.35	0.001

WBC = White blood cell, LYMPH = Lymphocyte, MXD = Mixed cell, NEUT = Neutrophil, NFR = Neutrophil function rate, TB = Total bilirubin, CB = Conjugated bilirubin, UB = Unconjugated bilirubin, GSH = Glutathione

Table 2. Selected indicators of inflammation and haemolysis among adolescents living with sickle cell anaemia and infected with malaria parasite.

Parameters	Hb SS subjects with no malaria infection (n=33)	Hb SS subjects with asymptomatic malaria infection (n=35)	p-value
WBC ($\times 10^9$/l)	9.93 ± 1.57	12.86 ± 2.45	0.001
LYMPH ($\times 10^9$/l)	4.57 ± 1.31	4.01 ± 1.21	0.073
MXD ($\times 10^9$/l)	1.30 ± 0.35	1.53 ± 0.26	0.003
NEUT ($\times 10^9$/l)	4.06 ± 0.90	7.30 ± 2.15	0.001
NFR (%)	28.18 ± 3.42	23.63 ± 4.04	0.001
TB (µmol/l)	30.20 ± 5.04	38.28 ± 7.63	0.001
CB (µmol/l)	13.41 ± 3.96	16.87± 3.23	0.003
UB (µmol/l)	16.80 ± 3.32	21.41± 4.83	0.001
GSH (µg/ml)	1.67 ± 0.34	1.24 ± 0.21	0.001

WBC = White blood cell, LYMPH = Lymphocyte, MXD = Mixed cell, NEUT = Neutrophil, NFR = Neutrophil function rate, TB = Total bilirubin, CB = Conjugated bilirubin, UB = Unconjugated bilirubin, GSH = Glutathione

Table 3. Pearson's correlation values for subjects with sickle cell anaemia

Parameters	Pearson's correlation (r)	p-value
Glutathione and total white cell count	-0.695	0.001
Glutathione and neutrophil count	-0.614	0.001
Glutathione and neutrophil function rate	0.380	0.001
Glutathione and unconjugated bilirubin	-0.352	0.003

DISCUSSION AND CONCLUSIONS

The present study on selected inflammatory and haemolytic indicators in subjects living with sickle cell anaemia in a malaria -endemic population was carried out among affected adolescents between 12-18 years of age who were attending Haematology Clinic at University of Calabar Teaching Hospital in Calabar, Nigeria. This study has revealed the impact of malaria infection on neutrophilic response in sickle cell anaemia. There is heightened neutrophilia with reduced function rate among malaria-infected sickle cell anaemia subjects. The contributions of white blood cells to the pathophysiology of sickle cell anaemia remains a subject of interest. Adhesive properties of these cells to the endothelium of the microcirculation have been understood to play important role in sickle cell crisis (19,20). Associations between polymorphonuclear leucocytosis and increased rate of early death, acute chest syndrome and stroke have been reported (21,22). Elevation of neutrophil concentration in the blood of patients with sickle cell anaemia has been attributed to mechanisms such as accelerated release of neutrophils from the bone marrow, decrease in the rate at which neutrophils leave the blood as well as demarginating of intravascular neutrophils (23).

Chronic haemolysis associated with sickle cell anaemia was evident in the study as unconjugated bilirubin values were on the high side. Malaria infection apparently contributed to the haemolytic episodes as much higher mean value was recorded in this category. A reversed pattern of finding was, however, recorded for glutathione level. Chronic haemolysis potentiates generation of reactive oxygen species, reduced antioxidant capacity and ultimately increased oxidative stress (24,25).

Glutathione depletion observed in this study correlated with increasing unconjugated bilirubin value and absolute neutrophil count but decreasing neutrophil function rate. This study has shown that reduced levels of glutathione and high bilirubin concentrations are markers of oxidative stress. This observation is consistent with the findings previously reported from the studied population (25).

Lowered immunity remains a challenge for sickle cell anaemia subjects living in malaria-endemic areas. This reveals an important intervention target for the transition from paediatric to adult care in the management of sickle cell anaemia.

The present study had the limitation of not following up the subjects. Further studies may adopt a longitudinal study approach and monitor these markers from childhood to adult life.

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ORIGINAL ARTICLE

New diagnostic biomarkers for celiac disease in Egyptian children: Cyclophilin A and Netrin-1

Moushira Zaki, Eman R Youness and Hala T El-Bassyouni

ABSTRACT

Background: Celiac disease is a chronic inflammatory disease of the small intestine. Cyclophilin A (CYPA) is a highly abundant protein in the cytoplasm of most mammalian cells. Netrin-1 is a laminin-related secreted protein that is broadly expressed in numerous tissues. Our aim was to determine the efficiency of both markers in the diagnosis of celiac disease and their relations to clinical findings.
Methods: This study was conducted on 50 children (mean age: 8±3.2 years) with celiac disease and 48 age and sex matched healthy controls. Circulating serum CYPA, Netrin-1, and anti-tissue transglutaminase antibodies were measured using ELISA kits.
Results: Both markers were significantly higher in celiac disease patients compared to controls. Patients presented with low birthweight in 5%, 15% of the patients were the offspring of consanguineous families, delayed milestones in 10%, abdominal pain in 35%, diabetes Type 1 was found in 10%, all patients had increased anti-tissue glutaminase levels, and upper endoscopy lesions in 25%. Significant positive correlations were noted between anti-tissue transglutaminase antibodies and both markers.
Conclusion: Both markers had good diagnostic performance for celiac disease among Egyptian children.
Keywords: Celiac Disease; Diagnosis; Cyclophilin A; Netrin-1.

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INTRODUCTION

Celiac disease is an autoimmune enteropathy described through changes in the intestinal mucosa due to gluten ingestion in genetically predisposed individuals. The three pillars used for its diagnosis are serum antibody detection, duodenal biopsy, and genetic study. Removing gluten from the diet is effective for most individuals, achieving clinical improvement, a progressive decrease in antibody titers, and recovery of the duodenal mucosa (1). The occurrence of celiac disease is growing, partially because of testing for, and improved recognition of, the disease (2). The criteria for celiac disease diagnosis are fluctuating, however, diagnosis in adults still count on the incidence of atrophied duodenal villous whereas the patient is on a diet including gluten, together with outcomes from examinations in serum. If proven scalable and accurate, analyses that identify tetramer complexes of gluten-HLA could be utilized in diagnosis done in the conditions of a gluten-free diet in absence of intestinal biopsy. Moreover, serum anti-tissue transglutaminase represents the most common diagnostic marker (3). Celiac disease patients also demonstrate the presence of alleles of HLA- DQ2 or HLA- DQ8, class II major histocompatibility complex as a genetic risk factor (4,5).

Recent improvements in the comprehension of celiac disease pathogenesis still emerging that may entangle the gluten exposure role. Numerous studies have revealed that the gluten quantity utilised by the child might impact on the age of onset of celiac disease in hereditarily predisposed persons. Novel recommendations from the European Society of Paediatric Nutrition, Gastroenterology, and Hepatology permit celiac diagnosis on serology, ignoring endoscopic biopsies for infants. Updated guidelines and latest information in adults do not favour biopsies for patients who are genetically vulnerable for celiac disease who have been identified via clinical signs of celiac disease with serology (6).

Cyclophilin A (CYPA) is a universally dispersed protein belongs to the family of immunophilin. CYPA has the activity of peptidyl prolyl cis-trans isomerase (PPIase), that controls protein trafficking and folding. CYPA largely activates pro-inflammatory pathways, and it is a direct chemoattractant for inflammatory cells. In addition, it enhances the production of reactive oxygen species and shares in increasing the proliferation of macrophages. High expression of CYPA associates with deprived outcome of patients with inflammatory diseases (7).

Netrin-1 is a neuronal regulator signal which controls cytoskeleton rearrangement, cellular migration, and activation in several cell kinds. It is a chemotropic protein expressed in tissues and stimulates both repulsive and attractive responses of migration (8). Netrin-1 regulates the immune response via the suppression of macrophage and neutrophil migration (9). Its expression is on vascular endothelium where it is regulated by inflammatory cytokines and infection. Netrin-1 acts as a powerful suppressor of migration to altered chemotactic stimulants in vitro and in vivo (10). These facts propose that expression of Netrin-1 endothelially might prohibit migration of basal cell to the tissues and that its down-regulation with the onset of inflammation/sepsis might help employment of leukocyte (11).

The development of new diagnostic biomarkers can provide a better noninvasive approach in the diagnosis of celiac disease. Further elucidations of the role played by CYPA and Netrin-1 on celiac disease will help in designing novel pharmacological therapies for this disease.

MATERIAL AND METHODS

This study comprised 50 children (mean age: 8±3.2 years) with celiac disease and 48 age and sex matched healthy controls. All celiac disease cases were on a gluten-free diet for more than six months. They were recruited from the outpatient clinics of the Clinical Genetics Department, National Research Centre, Egypt. The diagnosis was established based on clinical features, biochemical investigations, and upper endoscopy assessment. All samples were collected after obtaining the parents' informed consent using a form approved by the Ethical Committee of the National Research Centre (6).

Serum CYPA, Netrin-1 and anti-tissue transglutaminase antibodies levels were determined by ELISA (R&D Systems) according to the manufacturer's instructions.

Data are reported as mean ± standard deviation. For the comparison of normally distributed variables between groups, Student's t-test was used. Pearson's correlation coefficient was used to test the strength of any association between different variables. SPSS for Windows (SPSS Inc., Chicago, IL) was used. All tests were 2-tailed and statistical significance was set at the p0.05 level.

RESULTS

This study included 50 patients, 28 were females and 25 were males (1.12:1), Table 1 shows the characteristics of these patients. The mean levels of CYPA and Netrin-1 were increased in celiac disease patients compared to controls as shown in Table 2. Significant positive correlations were detected between anti-tissue transglutaminase antibodies and both CYPA and Netrin-1 levels in celiac disease patients (Table 3).

Table 1. The clinical characteristics of children with celiac disease.

Characteristics and variables	N=50
Female/male ratio	1.12:1
Consanguinity	15%
Low birthweight	5%
Delayed milestone	10%
Abdominal pain	35%
Diabetes mellitus type 1	10%
Anti-tissue glutaminase	100%
Upper endoscopy lesions	25%
Mean of anti-tissue glutaminase (U/ml)	36±12.55

Table 2. Levels of CYPA and Netrin-1 in CD patients and controls.

Biochemical markers	Group	Mean± Std. Deviation	P
CYPA (ng/mL)	Controls	6.26 ± 0.37	0.001
	Celiac disease	7.68 ± 0.72	
Netrin-1 (ng/mL)	Controls	145.49 ± 15.81	0.001
	Celiac disease	181.38 ± 19.13	

Table 3. Correlation between anti-tissue transglutaminase antibodies, CyPA, and Netrin-1 levels in celiac disease patients.

Biochemical markers	Pearson correlation	Anti-tissue transglutaminase antibodies
CYPA	r	0.919
	p	0.000
Nertin-1	r	0.611
	p	0.020

DISCUSSION

Celiac disease is a mediator for immune systemic syndrome initiated in hereditarily vulnerable persons via eating of gluten found in wheat and associated cereal grains. In adulthood, the intestinal biopsy sampled by endoscopy is the gold standard for celiac disease diagnosis (12). Generally, celiac disease prevalence varies from 4.5% among high-risk persons to 0.75% in subjects not-at-risk (13). High-risk persons comprise the relatives of celiac disease patients, adults or children with celiac disease related signs (i.e., constipation, abdominal pain and diarrhea), and adult or children individuals with celiac disease-related conditions (i.e., Down syndrome, infertility, osteoporosis, anaemia, Diabetes Mellitus Type 1) (14). Our findings found low birthweight in 5%, 15% of the patients were the offspring of consanguineous families, delayed milestones in 10%, abdominal pain in 35%, diabetes Type 1 was found in 10%, all patients had increased anti-tissue glutaminase and upper endoscopy lesions in 25%.

The syndrome is described by a variable grouping of clinical manifestations contingent on exposure to gluten in diet, the incidence of CD-specific antibodies in serum (anti-endomysium antibodies and anti-tissue transglutaminase (anti-TG2)), and diverse grade of enteropathy. The alleles of class II major histocompatibility complex was shown in celiac disease patients (15). Several studies have examined anti-TG2 autoantibodies production in patients' intestines with explicit celiac disease at diagnosis. Anti-TG2 autoantibodies deposited in the intestine were noticed in 100% of cases in adults with untreated celiac disease (16) . More changeability was described in pediatrics. Mucosal precipitates were recognized in 96 to 100% of celiac patients who were untreated (17). In children younger than 2 years of age this percentage is decreased (73%). Generally, the disease happens between 6 and 18 months of age, after the taking of weaning foods comprising prolamins. Cyclophilin B (CYPB) and Cyclophilin A (CYPA) are the best investigated members of the family targeting it to the endoplasmic reticulum(18). CYPA, a multifunctional protein, is known to be an inflammatory mediator that is released from different kinds of cells in response to inflammatory stimuli. Several studies have revealed that CYPA levels are increased in disorders associated with inflammatory conditions (19-21). Netrin-1 is a laminin-related released protein that is broadly synthesised in various tissues, comprising renal tissues. In previous studies Netrin-1 was shown to have a role in and acceleration of angiogenesis (22), growth and regulation of inflammation, and the migration of vascular endothelial cells tumor progression (23,24). In the present study results provided evidence for the role of Netrin-1 in celiac disease by showing an increase of its levels in patients compared to controls. A previous study in Egyptian patients with celiac disease and Type I diabetes reported genetic linkage of HLA genotypes and Egyptian celiac disease patients (25).

In conclusion, both CYPA and Netrin-1 markers have potential clinical efficacy in the diagnosis and follow-up of celiac disease among Egyptian children. Further studies are required to introduce these biomarkers into the traditional management of the celiac disease to avoid endoscopy, especially in pediatric patients.

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ORIGINAL ARTICLE

Retrospective study of N-methyl-D-aspartate glutamate receptor IgG testing outcomes at LabPLUS, Auckland City Hospital, New Zealand, 2015 – 2020, in a clinically demand-managed setting

Paul M Austin, Richard H Steele and Helena T Thompson-Faiva

ABSTRACT

Objectives: To review the outcomes of N-methyl-D-aspartate glutamate receptor (NMDAR) antibody testing conducted at LabPLUS over the period 2015-2020.

Methods: A data extract over the period was obtained detailing patient testing. Retrospectively, a proportion of the tested patient cohort had their clinical records accessed to determine their final diagnoses.

Results: 654 patients were tested which resulted in the identification of 24 NMDAR antibody positive patients. All positive patients met a case definition for NMDAR encephalitis. A review of 100 NMDAR antibody negative patients did not identify any that supported a case definition for NMDAR encephalitis. The neurologist vetted cohort was predominantly adult (80%) with higher numbers of females. Patients of both genders ≤45 years of age accounted for 88% of the antibody positive cases. There was generally high compliance (>75%) in obtaining CSF specimens for testing from within the NRA (local testing) region which was critical, as 30% of patients would have had a delayed diagnosis and most likely treatment if an independent serum specimen had been sent. The laboratory was able to consistently meet the clinically required KPI of same working day reporting turn around times over the six year period for cases of suspected NMDAR encephalitis.

Conclusions: Under the framework of neurologist gate keeping and liaison LabPLUS was able to implement and consistently deliver high value clinical results (antibody positive and negative) that allowed rapid treatment intervention when indicated which then translated into the best possible outcomes for patients with NMDAR encephalitis. The success of the mono-specific NMDAR antibody service allowed further neuroimmunology assays to be added to the diagnostic portfolio.

Key words: N-methyl-D-aspartate glutamate receptor, encephalitis, clinically-managed service, turn around time.

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INTRODUCTION

Anti-NMDAR (N-methyl-D-aspartate glutamate receptor) encephalitis is an autoimmune based syndrome with a progressive clinical course that can be treated. The syndrome affects females in a ratio of 4:1 over males, can occur at any age but peak incidence is seen in children and young adults (1). In approximately 60% of females with the disease aged 17 years or more, there is an underlying tumour (often unidentified) with ovarian teratoma being the commonest (2). The clinical presentation can be broken down into four phases:

1. Prodromal phase

In approximately 70% of patients the encephalitis commences with somewhat non-specific symptoms of fever, malaise, poor concentration, headaches, nausea, vomiting, and diarrhoea. This phase lasts from one to three weeks (3). The remaining phases can vary both in terms of sequence and severity.

2. Psychotic and / or seizure phase

Patients undergo behavioural and emotional changes including fear, apathy, depression, decreased cognitive skills, and psychosis (4). Ataxia and choreiform movements may be noted (5). Seizures (tonic-clonic) may also occur; the severity and management of which may require admission to intensive care facilities where monitored settings for cardiac and respiratory support are available.

3. Unresponsive phase

Patients are unable to follow verbal commands and may appear mute. Other symptoms can include maintaining gaze as if in a catatonic state, smiling inappropriately and stereotyped athetotic movements of the hands and fingers (6).

4. Hyperkinetic phase

This phase is characterised by autonomic instability manifesting with cardiac arrhythmia, hypotension, hypertension, hyperventilation, hyperthermia and hypothermia. Dyskinesias, extra-pyramidal signs and stereotyped motor automatisms (lip smacking, teeth clenching, grimacing, sustained jaw movements, and oculogyric crisis) may be observed (7). Autonomic dysregulation is more common in adults, whereas speech dysfunction is more common in children (4).

Once the diagnosis of anti-NMDAR encephalitis is confirmed, aggressive treatment using high dose corticosteroids, immunosuppression, intravenous immunoglobulin, plasma exchange and rituximab either sequentially or in combination is given. Where identified, resection of the tumour is performed (6).

With prompt aggressive treatment the prognosis is good with approximately 75% of affected patients experiencing complete or near complete recovery (3). In the setting of delayed or ineffective treatment, mortality rates can exceed 90% (1). Relapses can occur in 20-25% of patients at intervals ranging from three months to nine years after the initial presentation (8). The commonest symptoms of relapsing patients include speech dysfunction, psychiatric symptoms, seizures, and disturbances of consciousness and attention. Relapse rates are highest in patients not treated with immunotherapeutics in the first episode and those where tumour resection was not performed (3,9).

Prior to 2015 all neuronal antibody requests received at LabPLUS were sent overseas to multiple institutions, many of which were tested in research laboratories rather than accredited diagnostic laboratories. There was no clinical or technical oversight in the send away process leading to

inappropriate requesting, high service costs, and result turn around times (TAT) that exceeded the clinical relevance. It was against this backdrop that a planned on-site (LabPLUS) diagnostic neuroimmunology service was envisioned. The new service was demand-managed by neurologists acting as gatekeepers for all requested tests. Immunopathologists added value in discussions that were required for challenging cases as well as providing an interface for the diagnostic laboratory. For reasons outlined earlier in this introduction, NMDAR encephalitis was the first condition chosen to deliver on-site testing, not only to improve patient outcomes but also to demonstrate proof of concept of the new service which, if delivered, would then allow future expansion.

The Euroimmun Glutamate Receptor 3 Indirect Immunofluorescent (IIF) assay was initially verified for use by on-site (LabPLUS) testing of retained specimens (serum or CSF; N=35) that had been referred to Royal Brisbane Hospital, Australia or Oxford Hospital Laboratories, UK. Concordance with the overseas testing site was 100% (14 positive, 21 negative; unpublished data).

Following the verification, a 10-month pre-and post-implementation audit was conducted (98 patients pre-implementation; 106 patients post-implementation). The three main findings [unpublished data] were:

1. A significant decrease in result TAT from 25 days overseas to three days on-site.
2. Reduction of inappropriate requests as a direct result of the demand-managed process. The most significant change was seen from the Child and Family Unit, ACH where in the pre-implementation period 46 tests were sent overseas compared with four tests performed on site in the post-implementation audit period (91% decrease).
3. A significant improvement in taking the clinically appropriate specimen type. Pre-implementation CSF with or without serum: 36/114 – 31%; post-implementation CSF with or without serum 110 / 150 – 73%.

This retrospective review study was initiated to (a) determine if the improvements seen immediately after implementation had been retained consistently over time (six years); (b) to characterise our testing population on the basis of gender, age and site of referral; and (c) to establish the assay's overall performance in terms of both definitive diagnosis and exclusion utility.

MATERIALS AND METHODS

Euroimmun Glutamate Receptor 3 IIF assay

The principle of the assay is IIF where per microscope field (patient test) there are both transfected (NR1 subunit of the NMDAR complex in EU90 cells) and untransfected EU90 cells embedded. After initial specimen incubation and wash steps a goat anti-human IgG (ɣ-chain specific) –FITC reagent is added. After a further set of incubation and wash steps slides had cover slips applied and were viewed for characteristic (nuclear with cytoplasmic extensions) staining using Zeiss LED-based fluorescence microscopy (excitation: 470/40nm; emission: 515nm) at a magnification of X200 (Figure 1). Serum specimens were tested at dilutions of 1:10 and 1:50. CSF specimens were tested undiluted and at a dilution of 1:10. Phosphate-buffered saline was used as the specimen diluent.

Results are typically reported qualitatively (detected/not detected). However, the analytical and reporting system is sufficiently flexible to accommodate semi-quantitative testing and reporting in challenging cases.

Data extraction

Review period: 1 January 2015 – 6 November 2020. Note: year 2020 comprised 10 months data and testing numbers were impacted by the COVID-19 global pandemic.

Regional classification: Northern Regional Alliance (NRA): ADHB (Auckland City Hospital, Starship Children's Hospital), CMDHB (Middlemore Hospital), WMDHB (North Shore Hospital), NDHB (Whangarei Base Hospital, Dargaville Hospital, Bay of Islands Hospital, Kaitaia Hospital). Patients were identified as being within or having specimens referred for testing from outside the NRA.

Paediatric/adult age classification: Patients ≤ 15 years of age were classified as paediatric.; patients ≥ 16 years of age were classified as adult.

Patient diagnoses in NMDAR antibody positive (N=11) and negative cohorts (N=100) from within the NRA region: Diagnoses were determined by clinical chart review of medical records. Patient identities were not disclosed and data was used anonymously. As this was a retrospective study with no modification on clinical decision making or individual follow up, patient informed consent was not required.

RESULTS

Overview

In the review period, 654 patients underwent testing for mono-specific NMDAR encephalitis, averaging 109 patients per annum. Of the 654 patients tested, 24 cases of NMDAR encephalitis were identified (3.7%); range: 1.9% - 6.3%), positive cases being seen in every year testing was conducted (Figure 2). Of the 24 positive cases, 14 (58%) were female and 10 were male (42%). A single patient had confirmed NMDAR encephalitis historically and was admitted to hospital for on-going seizures, the remaining 23 cases were new diagnoses.

Further, in February 2017, the limbic encephalitis service was expanded (in addition to NMDAR antibody) to include the following five targets in a single test format: (a) leucine-rich glioma inactivated protein (LGI 1), (b) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA 1/AMPA 2), (c) B1 subunit of the ɣ-aminobutyric acid receptor ($GABA_B$), and (d) contactin associated protein 2 (CASPR 2). The testing is commonly referred to as the 'LE chip'.

A full review of testing outcomes from the LE chip will be presented in a subsequent paper. However, with direct reference to NMDAR antibodies, in the review period, 693 patients were tested using the LE chip with a further 3 positive cases identified (0.4%). With the inclusion of the LE chip testing, there were 27 positive cases out of 1,347 patients tested (2.0%). The data presented in this retrospective review is restricted to those patients whose specimens underwent testing using the mono-specific NMDAR antibody assay.

Patient age/gender characterisation

In every year, numbers of female patients exceeded males by an average of 1.4:1 across the six years. The average did not differ markedly from year to year (range: 1.2:1 – 1.6:1) (Table 1). Patients were predominantly adult across both genders (80% adult, 20% paediatric) over the 6 year period with little variation seen year to year (Table 1). Almost one-quarter (24%) of males and females were in the highest age size class (>61 years) (Table 1). The majority of NMDAR IgG positive cases were in patients ≤ 45 years of age (87.5%), with 62.5% of cases aged ≤ 30 years (Figure 3). In the same age bracket (0-30 years) patients tested accounted for 43.7% (286 / 654) of the total (Table 1, Figure 3).

There were approximately equal numbers of male and female NMDAR IgG positive paediatric cases (Table 2). There were over twice as many adult antibody positive cases with a higher (almost doubled) female predominance (11 F, 6 M) (Table 2). For antibody positive adult males, they were either in a relatively young age group (range: 22-27 years) or in older age group (range: 64-86 years) (Table 2). For antibody positive adult females, all cases were in a relatively tight age range of 18-42 years with a mode of 31 years (Table 2).

Specimen type site/referral

Approximately twice the numbers of paediatric patients were tested from within the NRA as opposed to outside the region and this was consistent year to year. Conversely, higher numbers of adults were referred for testing from outside the NRA at an overall ratio of 1.75:1 across the six years (Figure 4). It was noted that for the initial two years, referred adult specimen numbers from within and outside the NRA were approximately equal. However, over the period 2017 – 2019 external NRA referred specimen numbers were consistently higher (minimum three times) compared to those sent for testing from within the NRA.

Whether referred from within or external to the NRA there was very good compliance of taking a CSF specimen type (independently or with a paired serum) for investigations on paediatric patients which was consistent over the six year period (NRA – 68/89; average: 76%), External to NRA – 32/41 (average: 78%) (Figure 4). However, for adult patients from within the NRA, over the six year period while there was acceptable compliance in obtaining CSF specimens (133/190 (average: 70%: range: 54% - 82%), outside the NRA the overall frequency of taking a CSF specimen dropped to a low average of 49% (163/334) with a range of 30% - 57% (Figure 4).

Twenty-one of the twenty-four (87.5%) NMDAR IgG positive cases had a referred CSF (N=3) or paired CSF and serum (N=18) (Table 3). Of the 18 patients where a CSF and serum was collected, at time of testing, antibody reactivity was only seen in the CSF specimen in seven of the cases (39%).

Report turnaround time (TAT)

In this study, TAT is defined as the time (days) from specimen receipt to the issuing (electronic) of the report. For all NMDAR IgG antibody positive cases the requesting clinician was directly contacted on the day the report was issued. For the 24 antibody positive cases, the average TAT was 0.9 days with a range of 0 -2 days. Cases within the NRA (N=11) had a faster average TAT of 0.5 days (range 0-1 day) compared to those referred from outside the region (N=13; average; 1.1 day, range 0-2 days) (Table 3).

Confirmation of NMDAR encephalitis in an antibody positive setting

For the 23 patients with an undiagnosed acute encephalopathic picture that were NMDAR antibody positive, a formal diagnosis of NMDAR encephalitis was confirmed. Of the 23 new cases of NMDAR encephalitis, 10 were within the NRA region, seven being adult and three paediatric. The clinical records of all 10 cases were reviewed as follows:

For the adult cases, all seven patients had serum and CSF specimens taken and a single case (7) had isolated reactivity in the CSF specimen (15%). All patients had an acute/sub-acute (< three months) presentation with compatible limbic encephalopathic features (Table 4). A single patient (1/6; 17%) had an abnormal MRI, and an additional patient (1/2; 50%) had an abnormal EEG (Table 4). Elevations in either CSF white cell counts and/or protein levels were seen in 5/7 patients (71%) (Table 4). Two of the five (40%) female patients had ovarian tumours which were resected. All patients were treated with combinations of immunosuppressive drugs, IVIG, PLEX and Rituximab, and all demonstrated improved clinical outcomes (Table 4).

A single paediatric case was a patient who had a severe clinical course of NMDAR encephalitis nine years previously, and although rapid treatment was afforded significant developmental retardation and physical (foot and spine) deformities were secondary to the encephalopathy (9). The review was for an increasing frequency of seizures. Although NMDAR antibody was present in the patient's CSF there were no other clinical features or investigations that supported a flare or recurrence of the NMDAR encephalopathy. The seizures were controlled by increasing the dose of Valproate (Table 4).

For the remaining three new paediatric cases, two had serum and CSF specimens taken which were antibody reactive in both specimen types. The third patient's diagnosis was delayed due to parental non-consent for a LP procedure (11). For this patient two serum tests were negative for the antibody before seroconverting on a third bleed (11). All three patients had an acute/sub-acute (< 3 months) presentation with compatible limbic encephalopathic features (Table 4). No abnormalities were seen on MRI (2/2 patients); however, EEG abnormalities were seen in all cases (Table 4). Elevations in CSF white cell counts were seen in 2/2 patients (100%) (Table 4). All patients were treated with combinations of immunosuppressive drugs, IVIG, and Rituximab, and all demonstrated improved clinical outcomes (Table 4).

Table 1. Age and gender distribution of 654 patients tested at LabPLUS for NMDAR IgG 2015 – 2020.

	Male						Female					
	0-15	16-30	31-45	46-60	61+	Totals	0-15	16-30	31-45	46-60	61+	Totals
2015	4	6	1	13	10	34	10	16	11	8	6	51
2016	7	18	6	10	21	62	11	15	12	19	22	79
2017	13	11	9	8	23	64	16	11	19	13	16	75
2018	9	12	3	7	9	40	16	19	13	8	18	64
2019	10	12	2	7	10	41	10	14	14	12	15	65
2020	9	9	3	4	8	33	15	13	6	6	6	46
Totals	52	68	24	49	81	274	78	88	75	66	73	380

Exclusion of NMDAR encephalitis in an antibody negative result setting

Clinical records of 100 (37%) of the 269 NRA NMDAR antibody negative patients were reviewed. The proportion of adult to paediatric cases reviewed matched the frequency of that seen in the NMDAR antibody positive cases (i.e. 70% adult; 30% paediatric). The overall frequency of female (F) to male (M) records reviewed was 2:1, broadly in-line with that seen in the total review cohort of 654 patients. For the adult group the F:M ratio of records reviewed was 1.9:1. For the paediatric group, the F: M ratio of records reviewed was 2.3:1. Overall, 75% of the patients reviewed had a CSF specimen taken (adult N=50/70; 71%); paediatric (N= 25/30; 83%). None of the 25 patients who had a serum specimen only tested had any subsequent specimens taken for analysis.

The largest group (N=16; 16%) of patients were those that had an acute psychotic episode/hallucinations (Figure 5). Within this group approximately equal numbers had CSF specimens (N=9) versus independent serum specimens (N=7) provided for testing. Of those patients with an encephalopathic clinical presentation supported by imaging findings (N=9; 9%), six (67%) patients had either an autoimmune basis (N=4) or an infectious disease basis (N=2). For those patients with an autoimmune basis two were a limbic encephalopathy (LGI1 and GABA-B antibody mediated), one was NMOSD (MOG antibody mediated), and one was due to Hashimoto's thyroiditis (autoimmune mediated hypothyroidism).

Table 2. Gender and age characterisation of the 24 patients tested positive for NMDAR IgG at LabPLUS 2015 – 2020.

Paediatric (N=7)		Adult (N=17)	
Gender	**Age**	**Gender**	**Age**
Male	3	Male	66
Male	7	Male	86
Male	11	Female	18
Female	1	Female	18
Female	13	Female	19
Female	14	Female	21
Female	14	Female	30
Adult (N=17)		Female	31
Gender	**Age**	Female	31
Male	22	Female	32
Male	23	Female	37
Male	27	Female	38
Male	64	Female	42

Table 3. Referral site, Age group, Specimen type and reporting turn-around time [TAT] for 23 patients tested as NMDAR IgG positive at LabPLUS 2015 – 2020.

Patient	Referral site	Paediatric/ Adult	Specimen type	TAT (days)	Patient	Referral site	Paediatric/ Adult	Specimen type	TAT (days)
1	External	Adult	CSF	2	**13**	External	Adult	Serum and CSF	2
2	External	Adult	Serum	2	**14**	NRA	Paediatric	Serum and CSF	0
3	External	Adult	Serum	1	**15**	NRA	Adult	Serum and CSF	0
4	External	Adult	Serum and CSF	1	**16**	NRA	Paediatric	Serum and CSF	1
5	External	Adult	Serum and CSF	0	**17**	External	Paediatric	Serum and CSF	1
6	NRA	Adult	Serum and CSF	1	**18**	External	Paediatric	Serum and CSF	2
7	NRA	Adult	Serum and CSF	1	**19**	NRA	Adult	Serum and CSF	0
8	NRA	Adult	Serum and CSF	0	**20**	External	Adult	Serum and CSF	1
9	External	Adult	Serum and CSF	2	**21**	NRA	Paediatric	Serum and CSF	1
10	External	Adult	Serum and CSF	0	**22**	NRA	Paediatric	Serum and CSF	1
11	NRA	Adult	Serum and CSF	1	**23**	External	Paediatric	CSF	1
12	External	Adult	CSF	0	**24**	NRA	Adult	Serum and CSF	0

TAT: Specimen received to report issued.

Table 4. Clinical features, diagnostic investigations, treatments and outcomes of the 11 patients within the NRA region who were NMDAR antibody positive.

Case	Adult/ Paediatric	Gender	NMDAR antibody	Symptom onset	Clinical features	MRI	EEG	CSF WCC ($>5mm^2$) Protein (>0.45g/L)	CT	Treatment	Outcome
1	Adult	Female	Serum & CSF both positive	1 week	Confusion, psychoses, catatonia, agitation	Normal	Not performed	WCC: 7	No ovarian teratoma	Immunosuppression IVIG	Improved but ongoing cognitive impairment
2	Adult	Female	Serum & CSF both positive	2 weeks	Confusion, paranoia, respiratory failure, movement disorder	Normal	Normal	Protein: 0.47	No ovarian teratoma	IVIG Rituximab	Improved but secondary neurologic symptoms manifest
3	Adult	Male	Serum & CSF both positive	3 days	Confusion, behavioural changes, agitation	Consistent with encephalopathy	Not performed	WCC: 11	Not performed	Immunosuppression Rituximab	Fully recovered
4	Adult	Female	Serum & CSF both positive	2 weeks	Psychoses, catatonia, agitation	Normal	Not performed	Normal	No ovarian teratoma	Immunosuppression VIG PLEX Rituximab	Fully recovered
5	Adult	Male	Serum & CSF both positive	1 month	Cognitive impairment, ID co-morbidity	Normal	Not performed	Normal	Not performed	Immunosuppression IVIG Rituximab	Significant cognition improvement
6	Adult	Female	Serum & CSF both positive	1 week	Headaches, seizures, hallucinations	Not completed	Abnormal consistent with encephalopathy	WCC: 94	Ovarian teratoma present: resected	Immunosuppression IVIG Rituximab	Fully recovered
7	Adult	Female	Serum & CSF tested. CSF only positive	1 month	Hallucinations. Dysphagia, behavioural changes	Normal	Not performed	WCC: 37	Ovarian teratoma present: resected	Immunosuppression IVIG	Fully recovered
Case	**Adult / Paediatric**	**Gender**	**NMDAR antibody**	**Symptom onset**	**Clinical features**	**MRI**	**EEG**	**CSF WCC ($>5mm^2$) Protein (>0.45g/L)**	**CT**	**Treatment**	**Outcome**
8	Paediatric	Female	Serum & CSF both positive	2 weeks	Headaches, behavioural changes, seizures	Normal	Abnormal consistent with encephalopathy	WCC: 43	No ovarian teratoma	Immunosuppression IVIG Rituximab	Recovered but with occasional aggressive outbursts
9	Paediatric	Male	Serum & CSF both positive. In the first episode, CSF was antibody negative at completion of treatment	Refer clinical	Historical (9 years prior) severe NMDAR encephalopathy Foot and spinal deformity, seizures, developmental delay ongoing	Not performed	Not performed	Normal	Not performed	No evidence of NMDAR relapse – Immunosuppression withheld Valproate increased for seizures Orthopaedic surgeries for foot and spinal deformities	Marked developmental delay Mobility improved with surgical interventions Seizures controlled
10	Paediatric	Male	Serum & CSF both positive	2 weeks Transfer from out of NRA region	Ataxia, behavioural changes, loss of verbal skills, orofacial dyskinesia's	Not performed	Abnormal consistent with encephalopathy	WCC: 7	Not performed	Immunosuppression IVIG	On discharge improved mobility and behaviours but poor verbal skills
11	Paediatric	Male	Serum only tested and positive	1 month	Seizures, hallucinations	Normal	Mildly abnormal	Consent not given for LP	Not performed	Immunosuppression IVIG	Fully recovered

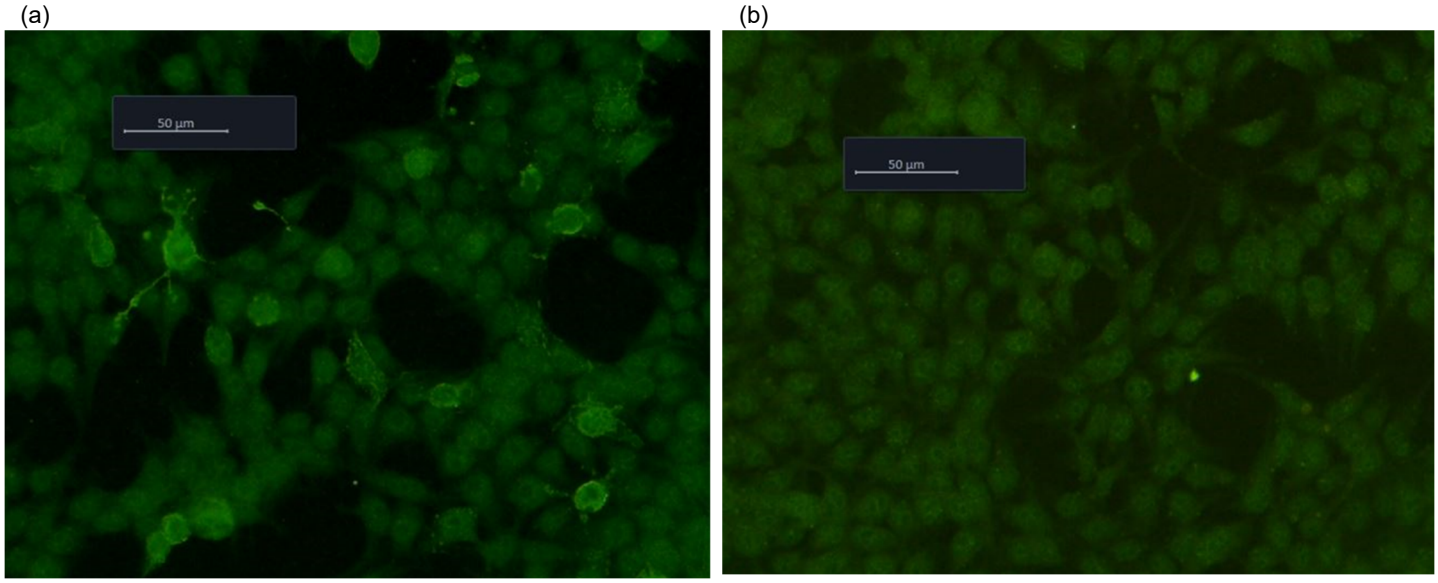

Figure 1. NMDAR antibody positive serum diluted 1:300, magnification x200 as expressed on Euroimmun (a) NMDAR transfected and (b) un-transfected EU90 cell line.

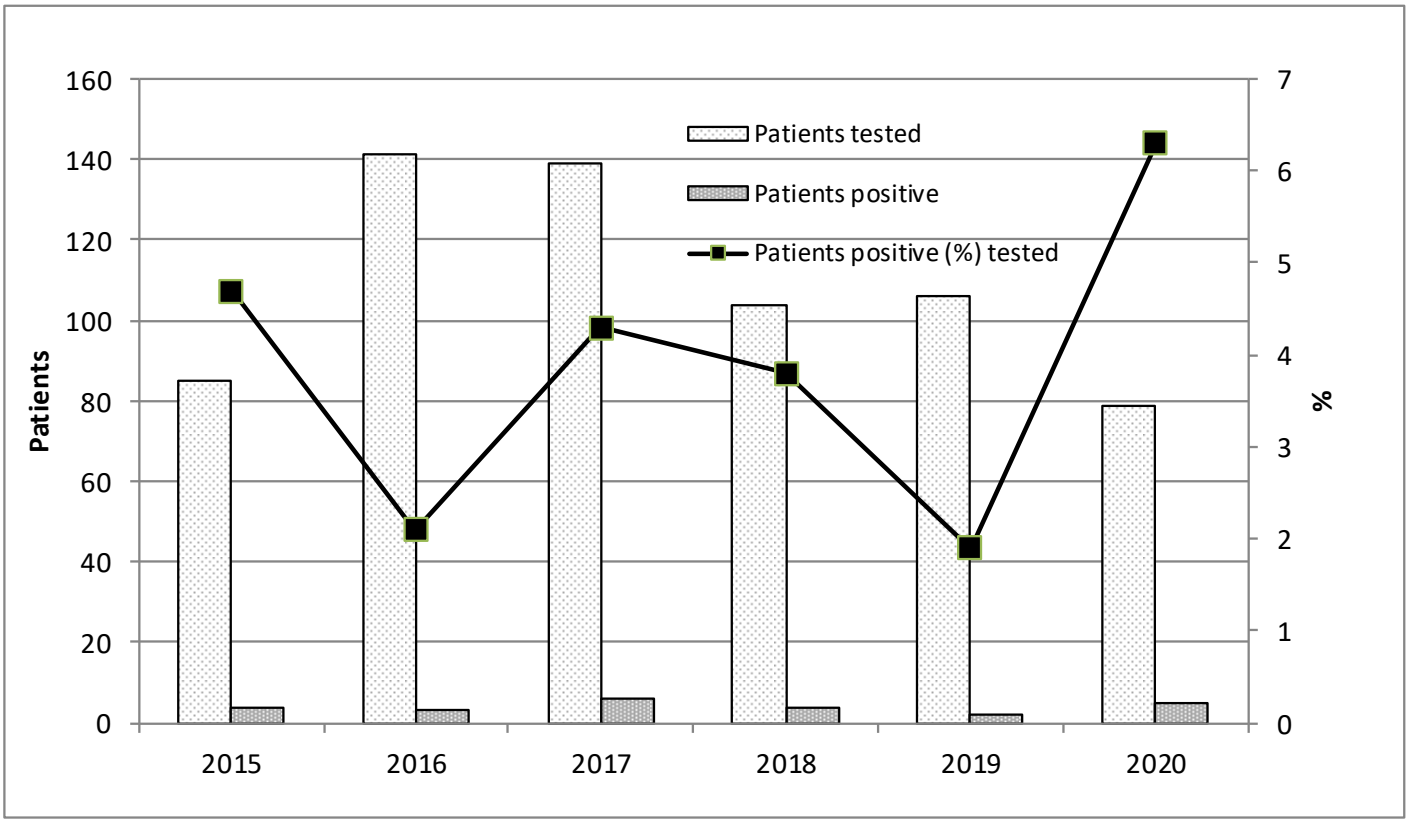

Figure 2. Frequency of NMDAR encephalitis cases identified at LabPLUS over a six year period (2015-2020) in a demand-managed neuroimmunology diagnostic service.

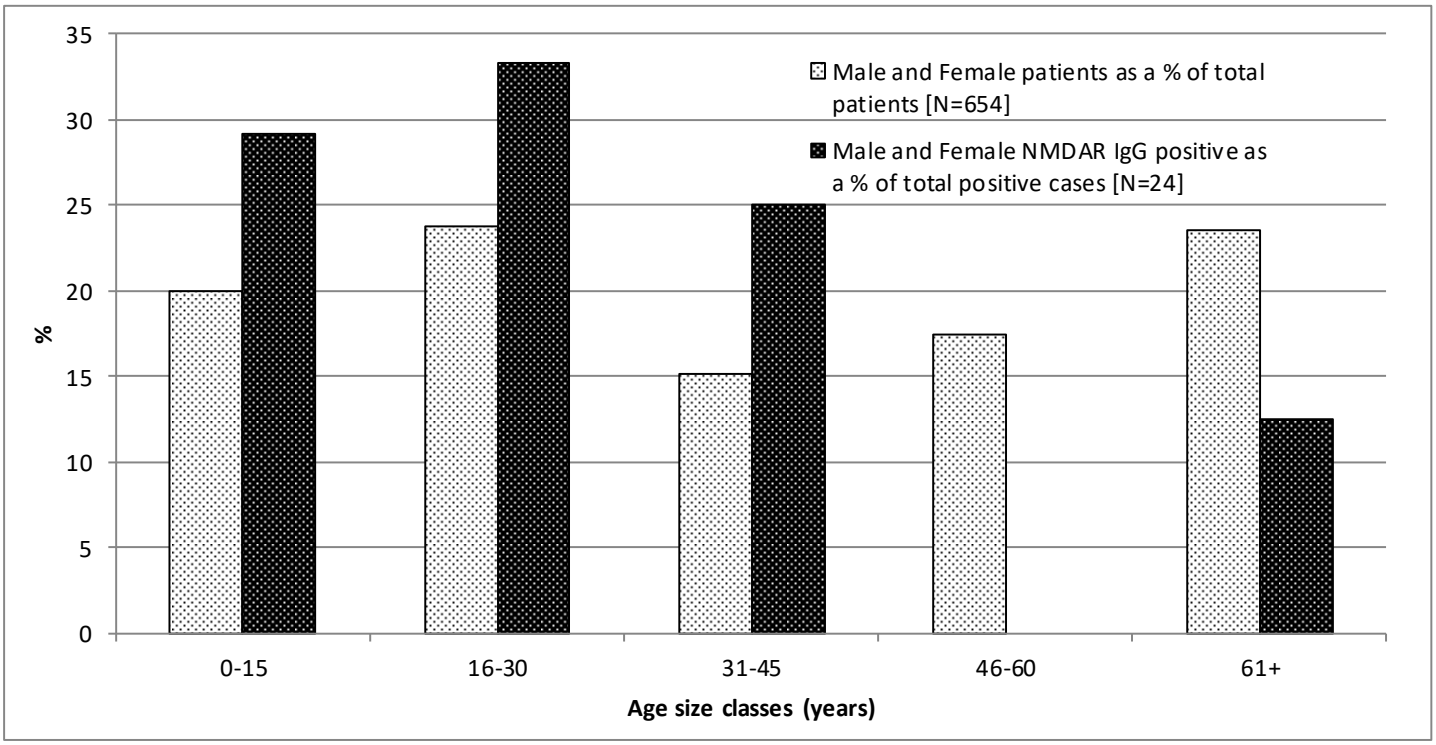

Figure 3. Age distribution of combined male and female patients tested with an added distribution of NMDAR IgG positive patients (combined male and female) represented as a proportion of total NMDAR IgG positive cases over the period 2015 – 2020.

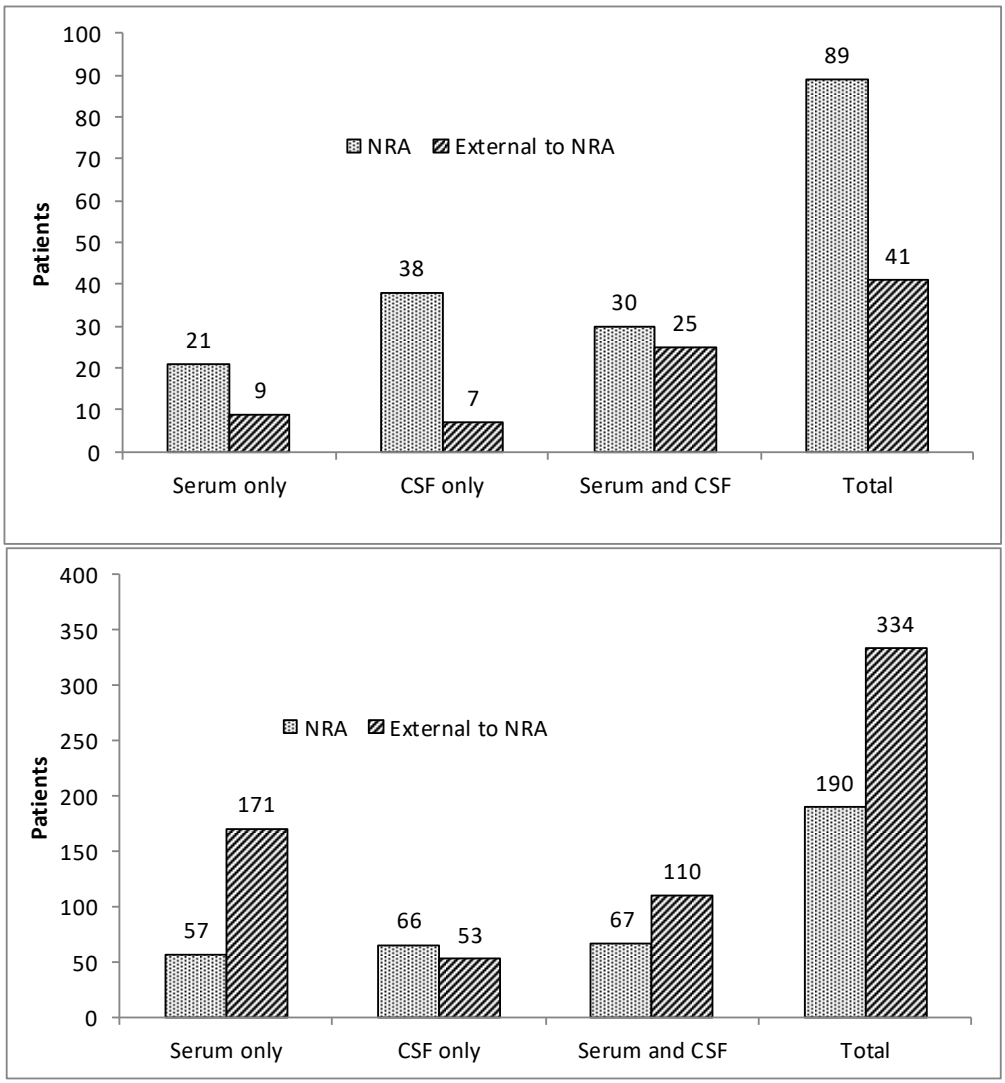

Figure 4. Distribution of referral site and specimen type for (a) paediatric and (b) adult patients tested for NMDAR IgG antibody at LabPLUS 2015 – 2020.

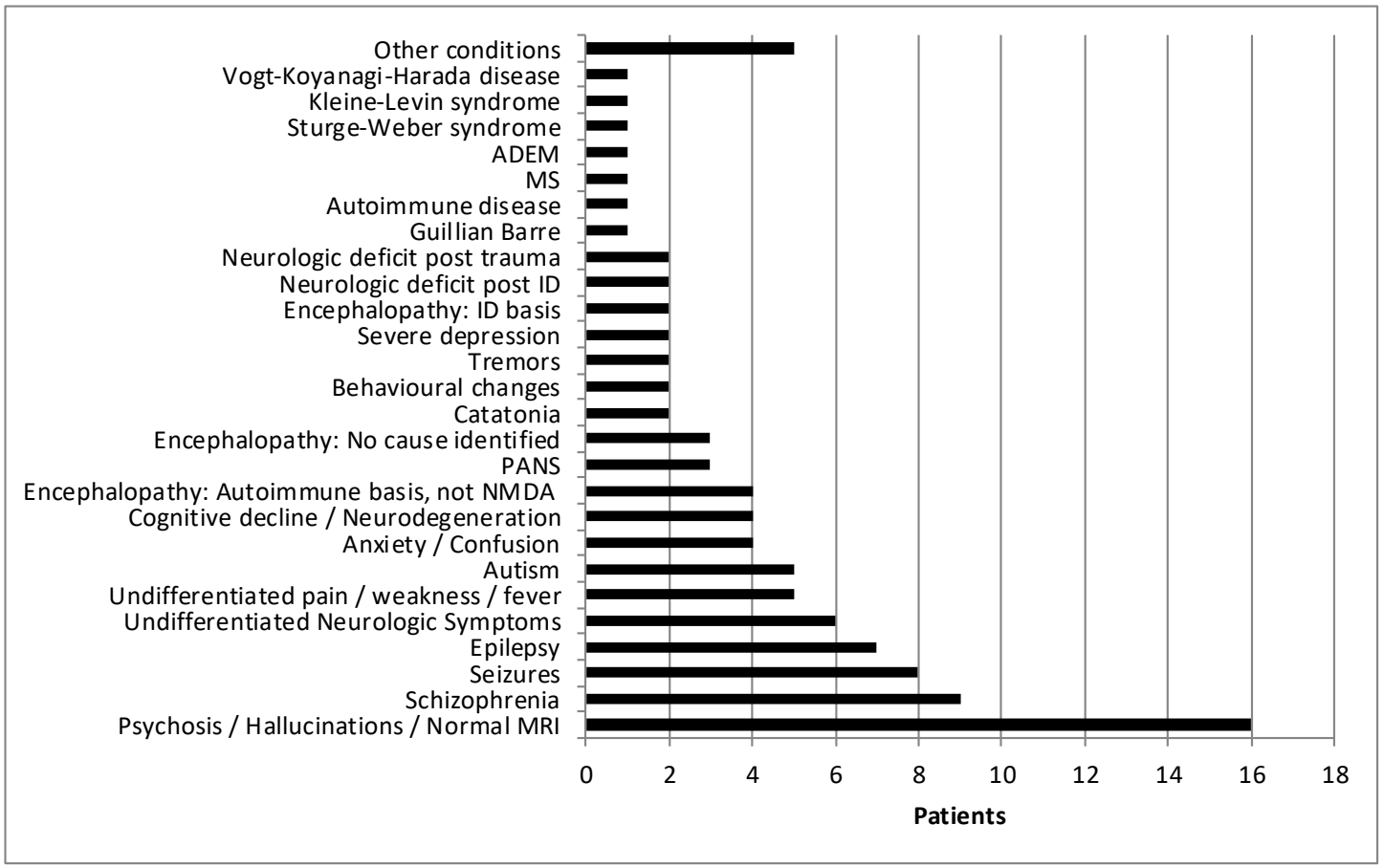

Figure 5. Confirmed diagnoses of 100 NMDAR antibody negative patients tested in the NRA over the period 2015 – 2020.

DISCUSSION AND CONCLUSION

Before discussing the sentinel findings from this retrospective analysis, it is also important to relate the body of work to the implementation of the first New Zealand diagnostic neuroimmunology testing service where NMDAR antibody testing was the flagship assay launched in 2015. The functional centrepiece of the service was to ensure neurologists acted as gatekeepers for all requesting.

The variety of symptoms from patients presenting with suspected autoimmune encephalitis, (AE) and in particular suspected NMDAR encephalitis, often pose significant challenges for those clinical teams that afflicted patients first encounter. An additional challenge for treating clinicians in non-neurological specialty is the regularly modified investigative algorithms that differ for adult and paediatric patients (10-11). The use of neurologists (adult and paediatric) in a gate keeping role was implemented to standardise the clinical approach to diagnoses. Tangible benefits that were expected to accrue were (a) that requests were appropriate and that the laboratory would not be overwhelmed with a non-specific volume of requesting; (b) that depending upon the investigation correct specimens were taken; (c) that assay TAT's were established on the basis of clinical need; (d) that communication lines with the pathology laboratory were robustly maintained and improved over time; (e) that neurologist involvement (diagnosis and treatment) was maximised for cases, the physicians having been at the forefront of the decision-making process; and (f) that the service was both fiscally viable and expandable. The expectation being that all of these stated benefits would then translate into improved patient outcomes.

From the original service planning meetings it was envisaged that if LabPLUS were able to deliver an appropriate service level for NMDAR antibody testing their neuroimmunology assay portfolio would expand over time. The hoped for service expansion was a rigorously planned exercise taking into account (a) the sequence of implementation of additional assays based upon clinical demand; (b) staff training requirements; (c) equipment procurement; (d) performance of technical validation studies with neurologist acceptance; and (e) IS resource availability. The authors fully understood that during the service expansion operational and performance standards for assays that had been implemented could not be allowed to be compromised.

The geographic site (within or external to the NRA) of referral of patients for testing was strongly associated with patient age. For adults, there was a ratio in excess of 2:1 for referrals outside the NRA region. For paediatric patient, there was still a 2:1 ratio but highest numbers were from within the NRA region.

As a direct consequence of the demand-managed service, in partnership with the neurologist gatekeepers, the laboratory was able to identify 24 cases of NMDAR encephalitis from 654 approved requests over the six year term giving an average frequency of disease in our tested population of 3.7% per annum. An article published in 2019 by Dalmau *et al.* stated that NMDAR encephalitis is a rare disorder with an incidence of 1-5 cases per million populations (12). Although this article is not intended to determine the frequency of NMDAR encephalitis in the New Zealand general population, the relatively high rate of antibody detection demonstrates the benefit of neurologist engagement ahead of the undertaking of the analytical procedure. Furthermore, the much higher rate (approximately 10 times) of antibody detection between requests for monospecific NMDAR (3.7%) versus requests for limbic encephalitis (0.4%) in roughly equivalent numbered patient cohorts is likely related to NMDAR encephalitis being the best characterised and most common form of limbic encephalitis (13). There is clear evidence from this study that in a demand managed service setting, with neurologist oversight, the provision of both mono-specific NMDAR as well as LE panel antibody testing is both appropriate and necessary.

The gender ratio of our tested population was 80/20 in favour of adults over paediatric patients and, somewhat surprisingly, there were similar numbers of males and females. For females tested numbers were consistent at approximately 20% of the total (N=380) per 15 year age bracket (years 0->61). By comparison the male tested population (N=274) demonstrated a bi-modal pattern with the initial peak being ages 0-30 years and then a secondary peak of ≥46 years age. Combined male and females (385/654; 59%) were aged ≤ 45 years of age, this age bracket accounting for 21/24 (88%) of NMDAR antibody positive cases. Seven of the twenty-four (30%) NMDAR antibody positive cases were from paediatric patients. Our data of a higher female incidence and prevalence in younger adults is broadly in line with published data (13) although our female prevalence was lower at a ratio of approximately 2:1, half of that which is generally quoted. This difference may be attributable to a relatively small antibody positive cohort (N=24). NMDAR encephalitis cases do occur in the older aged adults albeit at a very low frequency. In an observational cohort study by Titulaer *et al*. in 2013, 8/661 (1.2%) cases of NMDAR encephalitis were seen in patients aged ≥ 61 years (male N=4, range 62 -76 years; female N=4, range 62 – 85 years) (14). By comparison our cohort was entirely male at a much higher frequency of 12.5% of all antibody positive cases. Our higher frequency may be due to the fact that as patients age disease severity lessens (14). Patients with mild symptoms not seen by clinicians with specific expertise in autoimmune neurologic disease may not consider testing for NMDAR antibody potentially resulting in undiagnosed and/or mis-diagnosed cases.

From the NRA region 269 patients were reported as NMDAR antibody negative and overall 629/654 (96%) were reported as antibody negative. The obvious question to be posed is what is the exclusion value of a negative antibody result for NMDAR encephalitis? There are a number of published reviews and guidelines stressing the importance of testing CSF specimen type with or without a serum specimen as opposed to an independent serum specimen type due to higher comparative sensitivity of CSF during the acute stage of the disease (15-16). Of great concern was a published retrospective study by Guasp *et al*. in 2020 where, from 489 cases of confirmed NMDAR encephalitis, 75 (15%) patients returned a negative antibody test from an independent serum specimen. For those 75 patients the median time interval from symptom onset to antibody testing of 30 days was not statistically different to the 414 patients (35 day interval) who were seropositive on both CSF and serum specimens (16). The 75 seronegative patient subset were typically older and of male gender (16). This finding implies that use of an independent serum specimen will be challenged both by lack of sensitivity in the acute stage of the encephalopathy but also potentially in a subset of typically older males with the disease.

In our tested population, of the 23 patients who presented acutely with clinical features suggestive of NMDAR encephalitis, 20 had both a paired serum and CSF specimen taken. Six of the twenty patients (30%) had isolated reactivity in the CSF and as such, for these patients diagnosis and possibly treatment would have been delayed if only a serum specimen had been taken for antibody testing.

Clinical records were reviewed to determine the final diagnoses from 100/269 (37%) NRA region patients who were reported as antibody negative. The adult to paediatric split was 70/30 (patients) with twice as many female records reviewed compared with males. The proportions of adult/paediatric patients and female/male patients were in-line with the complete population. Overall, 75% of the 100 patient subset had a CSF specimen taken with or without serum (adult patients: 50/70, 71%; paediatric patients: 25/30, 83%). None of the 25 patients, where an independent serum specimen was originally taken, had any follow up specimens (CSF or serum) sent for analysis. Additionally, none of this group had a final diagnosis that was consistent with NMDAR encephalitis. On the basis of this subset patient review we believe that when NMDAR antibody results were reported as negative from our testing facility, particularly when the specimen type was CSF, that reported result had a very high likelihood for excluding NMDAR encephalitis as the pathology causing the patient symptoms.

Patients with NMDAR encephalitis will undergo a stage of psychosis which is variable from patient to patient in terms of severity, duration, and presentation within the constellation of symptoms associated with the disease (5,10). In our study we identified 5/10 (50%) acutely presenting confirmed NMDAR encephalitis patients from within the NRA region having psychoses as a component of their clinical presentation. Within the group of 100 NRA patients with negative NMDAR antibody results, 17 (17%; adult male N=4, adult female N=6, paediatric male N=2, paediatric female N=5) had psychotic symptoms. A CSF specimen was taken for 10 (59%) of the patients. None of the patients (a) had a final diagnosis of an encephalopathy; (b) had any follow up investigations; or (c) had any immunotherapy administered.

The now well recognised clinical stage of psychosis has led to an increased frequency of test requests for NMDAR antibody from psychiatrists in the setting of a patient presenting with a first episode psychosis. The 2016 Australian and New Zealand guidelines for investigations to be conducted for individuals presenting with first episode psychosis recommends testing for NMDAR and other antibodies (17). In the documented test listing and accompanying text there was an absence of commentary (a) regarding preferred specimen type; (b) the risks in the interpretation of results from independent serum specimens; and (c) regarding seeking advice from neurologists with expertise in autoimmune-based diseases. In two recently published articles the frequency of NMDAR antibody was determined by testing patients presenting with FEP (18,19). Across both studies the frequency of serum-based NMDAR antibody was 2.5% (11 antibody positive/441 patients). However, only four of the 11 antibody positive patients had either classical NMDAR encephalopathy symptoms (including the presence of ovarian teratoma) or were responsive to immunotherapy (18-19). Assuming all of the reactive reported antibody results were specific for the receptor, the implication is that serum antibody testing in such populations may give reactive results that are not associated with the well characterised encephalopathy.

With direct reference to the clinical and laboratory criteria for a definitive diagnosis of NMDAR encephalitis proposed by Graus *et al*. in their position paper in 2016 (10), all 23 patients that were NMDAR antibody positive on CSF and/or serum specimens met the case definition. It should be noted that the difference in the criteria for a diagnosis of AE versus NMDAR encephalitis is the (a) addition of MRI abnormality for AE; (b) the specific removal of reference to the presence of teratoma in AE; and (c) the replacement of reference of NMDAR antibody with detection of cell-surface, synaptic or onconeuronal antibodies for AE (10). Abnormal EEG or elevations in CSF white cell counts were specifically stated criteria for both AE and NMDAR encephalitis. For our 10 new diagnoses of NMDAR encephalitis seen in the NRA region, 4/5 (80%) patients had abnormal EEG findings, 6/9 (67%) patients had CSF pleocytosis, and 3/3 (100%) patients had an abnormal EEG and pleocytosis. Two out of four patients (50%) that only had a CSF investigation returned a normal white cell count. A single patient out of four (25%) that had both CSF and EEG investigations returned normal results for both. Interestingly, MRI investigations were performed on eight of the patients; a single patient (12%) returned an abnormal MRI. This finding endorses the recommendation from Graus and colleagues that abnormal MRI findings be restricted to the criteria for AE and not be included in the specific criteria for a case definition of NMDAR encephalitis.

Because of the clinical criticality for both diagnosis and initiation of treatment, our result TAT [defined as time from specimen receipt to time of issuing of the report] for any suspected case of AE is same day if the specimens are in the analytical area by 1000 hours. We were within this KPI for all 24 cases of NMDAR encephalitis and quicker if the patient was from within the NRA region. This consistently delivered speed

of service (which includes re-testing for new cases) allows physicians to either deliver or withhold immunotherapy with confidence. Apart from the electronic reporting facility, common in most if not all modern laboratories, for all antibody positive cases we additionally directly notify the requesting neurologist involved with the case immediately on completion of the testing. This last service feature ensures the rapid delivery of an appropriate treatment intervention irrespective of the clinical unit the patient is under admission to.

In summary, the findings from this retrospective study clearly demonstrate the benefit of having pre-analytical vetting of requests and familiarity of potential positive cases by neurologists. Within the framework of pre-analytical vetting, in conjunction with highly trained medical laboratory scientists supported by immunopathologists, we consistently deliver high value results within the clinically demanded time-frame for cases of suspected NMDAR encephalitis. The networking that has developed over time between the laboratory and the neurology teams throughout the country has ensured that for the majority of patients we now see from within the NRA region have CSF specimens taken for antibody testing. In the early years of the service, specimens referred from outside the NRA region were usually serum, however, due to the afore-mentioned networking efforts a higher proportion of CSF specimens are now being received. The high degree of specimen (CSF) compliance, speed of service and application of pre-test probabilities by neurologists all come together to maximise the benefit to the patients under investigation. Feedback from both adult and paediatric neurologists for the service that has been developed and delivered is uniformly positive and collegial. The success story of the mono-specific NMDAR antibody testing allowed the further development of the neuroimmunology diagnostic service that is now offered at LabPLUS.

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ORIGINAL ARTICLE

A two-year study of microbiological characteristics of intravascular catheter-related bloodstream infections at Razi Hospital, Iran

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ABSTRACT

Objectives: A substantial proportion of healthcare-associated infections are typically associated with devices such as indwelling intravascular devices (arterial and venous catheters), resulting in increased long-term hospitalisation, cost, morbidity, and mortality. This study aimed to determine the microbiological characteristics of bloodstream infection caused by intravascular device catheters in the north of Iran.

Methods: This retrospective study was conducted between 2018 and 2019 on inpatients with catheter-related bloodstream infections. Bacterial isolation and identification were carried out using standard microbiological and biochemical techniques. The disc diffusion method was used to determine antimicrobial susceptibility.

Results: Out of 287 examined catheters, 95 (33.1%) cases were positive for significant bacterial growth. Catheter-related bloodstream infections were most frequently caused by coagulase-negative staphylococci (28.4%), *Staphylococcus aureus* (15.8%), *Klebsiella pneumoniae* (14.7%), and *Pseudomonas aeruginosa* (12.6%). According to antibiotic susceptibility testing, the most effective antibiotics against staphylococci were amikacin, co-trimoxazole, and tetracycline. Meanwhile, 33.3% of *S. aureus* isolates and 56% of coagulase-negative staphylococci were methicillin-resistant. Gram-negative isolates showed a very high rate of antibiotic resistance, even toward the last resorts antibiotics such as carbapenems.

Conclusions: Our study revealed an alarming rate of catheter-associated infection, necessitating implementing a more stringent and effective infection control policy. Additionally, our observations provide critical data for making more targeted empirical antibiotic selections based on the local antibiotic susceptibility pattern.

Key words: Healthcare-associated infection; intravascular catheter-related infections; antibiotic resistance; bacterial infection.

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INTRODUCTION

Nosocomial infections, alternatively referred to as healthcare-associated infections, occur during receiving health care in a hospital or other health care facility where the patient was not admitted (1). Adverse drug reactions, healthcare-associated infections,, and surgical complications are the most frequently encountered adverse events in hospitalised patients (2). More than half of all reported healthcare-associated infections originate in developing countries (3). Bacteria are responsible for most healthcare-associated infections while mycobacterial, viral, fungal, and protozoal agents are less frequently implicated (2).

Staphylococci, a diverse group of *Enterobacteriaceae* and *Pseudomonas* spp. along with *Acinetobacter* spp. are the most common bacteria that cause healthcare-associated infections. These infections account for up to 70% of all ICU infections (2). Pneumonia, urinary tract infection (UTI), and bloodstream infection are the most common types of healthcare-associated infections. Furthermore, surgical site infection, gastroenteritis, and meningitis are also prevalent (2).

A substantial proportion of healthcare-associated infections is typically associated with the use of indwelling intravascular and urinary catheters, which can increase long-term hospitalisation, cost, morbidity, and mortality (4). The method and site of catheter insertion and underlying diseases are the primary risk factors for catheter-related infections (5). Biofilm formation is a common bacterial strategy for survival, which is a critical concern for public health (6).

Colonisation on the tip, internal, or external surfaces of catheters are essential for infection durability, as bacteria associated with biofilms exhibit significantly reduced susceptibility to antibiotic therapy (6). A significant impediment to treating healthcare-associated infections is the global rise in antibiotic-resistant organisms. As a result, applying methods to prevent nosocomial infections is gaining momentum (7).

Monitoring healthcare-associated infections is a vital and primary step in preventing and controlling infection widely accepted worldwide (8). By implementing strategies to prevent catheter-associated infections, health care costs, morbidity, and mortality can be significantly reduced. The purpose of this study was to determine the prevalence, bacterial aetiology, and pattern of antibiotic resistance in intravascular catheter-related infections in northern Iran.

METHODS

Study design and population

We conducted a retrospective study on patients hospitalised with catheter-related bloodstream infections at Razi Hospital, a teaching and rehabilitation facility in northern Iran. The purpose of this study was to evaluate patients admitted to Razi University Hospital with arterial or venous catheterisation in 2018 and 2019. In the absence of other sources of blood stream infection, an episode of catheter-related bloodstream infection was defined as catheters inserted for at least ≥48 hours and with at least one positive culture obtained from a patient with clinical manifestations such as fever or hypotension. The study design was approved by the Ethics Committee of the Guilan University of Medical Sciences (Reg No. IR.GUMS.REC.1398.392) and followed the declaration of Helsinki.

Sample processing and bacterial identification

Each patient's catheter site was cleaned before sampling and the catheter was removed without contacting the skin. The catheter's terminal 5 cm was cut into a sterile container and transferred to the laboratory within one hour. Following that, all catheter tips were transferred to 1 mL tryptic soy broth for culture. Then, 10 µL of bacteria growth in tryptic soy broth was transferred to blood agar, chocolate agar, and MacConkey

agar. The blood agar and MacConkey agar plates were incubated aerobically at 37ºC for 24-48 hours. At 37 ºC, chocolate agar plates were incubated in an atmosphere supplemented with carbon dioxide (a candle jar), and plates with a high level of bacterial growth were chosen for further processing. To identify positive samples standard microbiological and biochemical methods were used to test isolates, including morphological analysis, Gram staining, catalase, oxidase, coagulase tests, sugar fermentation, and other phenotypic biochemical tests.

Antibiotic susceptibility testing

The disc diffusion method was used to determine antimicrobial susceptibility on Mueller-Hinton agar (Merck, Germany) following the Clinical and Laboratory Standards Institute (CLSI) recommendations (9). CLSI recommendations were followed to select antimicrobial discs (Padtan Teb, Iran), control strains, and interpretation of results for each pathogen.

Statistical analysis

For statistical analysis, the SPSS™ software (version 21) was used. The findings are presented in the form of descriptive statistics based on relative frequency. Frequencies and percentages were used to summarise categorical variables, while median and interquartile range (IQR) values were used to describe continuous variables.

RESULTS

During the study period, a total of 287 intravascular catheters (102 in 2019 and 185 in 2020) were removed from hospitalised patients and sent to the laboratory. From the tested catheters, 95 (33.1%) were positive, demonstrating significant bacterial growth. Infected patients' median (IQR) age was 65 (52-73), ranging from 19 to 95.

Catheter-related bloodstream infections were caused by a total of 12 different types of bacteria, with 46 (48.4%) cases caused by Gram-positive bacteria and 49 (51.6%) by Gram-negative bacteria (Table 1). Coagulase-negative staphylococci were the most prevalent bacteria (28.4%), followed by *Staphylococcus aureus* (15.8%), *Klebsiella pneumoniae* (14.7%), and *Pseudomonas aeruginosa* (12.6%).

Tables 2 and 3 illustrate the antibiotic resistance patterns of Gram-positive and Gram-negative bacteria, respectively. Amikacin, co-trimoxazole, and tetracycline were the most effective antibiotics against staphylococci, the most common cause of catheter-related bloodstream infections. Meanwhile, 33.3% of *S. aureus* isolates and 56% of coagulase-negative staphylococci were methicillin resistant. Gram-negative isolates showed a very high rate of antibiotic resistance, even toward the last resorts antibiotics such as carbapenems.

Table 1. Frequency of causative organisms isolated from CRBSIs.

Gram stain	Bacteria type	Frequency	Percent
Gram-positive	Coagulase-negative staphylococci	27	28.4
	Staphylococcus aureus	15	15.8
	Micrococcus spp.	3	3.2
	Enterococcus faecalis	2	2.1
	Streptococcus group D	1	1
Gram-negative	*Klebsiella pneumoniae*	14	14.7
	Pseudomonas aeruginosa	12	12.6
	Acinetobacter baumannii	7	7.4
	Enterobacter cloacae	5	5.3
	Escherichia coli	4	4.2
	Citrobacter spp.	4	4.2
	Stenotrophomonas maltophilia	1	1
Total		95/287	33.1

Table 2. Antibiotic resistance pattern of Gram-positive bacteria*

Antibiotics	CoNS (N = 27) %			*S. aureus* (N = 15) %			*Micrococcus* (N = 3) %			*E. faecalis* (N = 2) %		
	S	I	R	S	I	R	S	I	R	S	I	R
Penicilin	0	0	100	8		92	0	0	100	0	0	100
Ampicilin	-	-	-	-	-	-	-	-	-	50	0	50
Cefoxitin	44	0	56	66.7	0	33.3	-	-	-	-	-	-
Tetracycline	54	0	46	46	0	54	-	-	-	50	0	50
Amikacin	58	0	42	61	8	31	-	-	-	-	-	-
Gentamicin	44	7	48	53	7	40	-	-	-	0	0	100
Clindamycin	27	0	73	33	0	67	0	0	100	-	-	-
Erythromycin	13	0	87	29	0	71	0	0	100	-	-	-
Ciprofloxacin	21	0	79	33	0	67	-	-	-	-	-	-
Co-trimoxazole	54	0	46	64	7	29	-	-	-	0	0	100
Vancomycin	-	-	-	-	-	-	-	-	-	50	0	50

*Results estimated based on the numbers of tested isolates.

Table 3. Antibiotic resistance pattern of Gram-negative bacteria*

Antibiotics	*K. pneumonia* (N = 14) %			*P. aeruginosa* (N = 12) %			*E. cloacae* (N = 5) %			*E. coli* (N = 4) %			*Citrobacter* (N = 4) %			*S. maltophilia* (N = 1) %			*A. baumannii* (N = 7) %		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Piperacilin	0	0	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Piperacilin-Tazobactam	25	0	75	0	0	100	0	50	50	0	33	67	33	0	67	-	-	-	0	0	100
Cefazolin	0	0	100	-	-	-	0	0	100	0	0	100	-	-	-	-	-	-	-	-	-
Cefoxitin	0	0	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cefotaxime	20	0	80	-	-	-	0	0	100	0	0	100	-	-	-	-	-	-	0	0	100
Ceftazidime	17	0	83	29	0	71	33	0	67	-	-	-	100	0	0	0	0	100	0	0	100
Ceftriaxone	0	0	100	-	-	-	-	-	-	0	0	100	0	0	100	-	-	-	-	-	-
Ceftizoxime	-	-	-	0	0	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cefepime	22	0	78	0	0	100	0	0	100	0	0	100	33	0	67	-	-	-	0	0	100
Tetracycline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	100
Amikacin	21	0	79	46	0	54	-	-	-	25	0	75	25	0	75	-	-	-	0	29	71
Gentamicin	23	0	77	40	0	60	25	0	75	25	0	75	0	0	100	-	-	-	33	17	50
Ofloxacin	0	0	100	100	0	0	0	0	100	-	-	-	-	-	-	-	-	-	-	-	-
Ciprofloxacin	27	0	73	43	0	57	50	25	25	25	0	75	50	0	50	100	0	0	14	14	71
Meropenem	0	0	100	0	0	100	50	0	50	0	0	100	33	0	67	-	-	-	-	-	-
Imipenem	22	0	78	75	0	25	67	0	33	0	33	67	100	0	0	-	-	-	17	0	83
Co-trimoxazole	18	9	73	-	-	-	60	0	40	25	0	75	25	0	75	100	0	0	25	25	50

*Results estimated based on the numbers of tested isolates.

DISCUSSION

Catheter-related bloodstream infections are widely regarded as one of the most severe health system challenges of the modern era, owing mainly to the increased use of venous catheters in recent years (5,10). We examined the prevalence and aetiology of catheter-related bloodstream infections in hospitalised patients in this study. Out of 287 studied catheters, 33.1% had positive culture, indicating the presence of catheter-related bloodstream infections. Our study's prevalence of catheter-associated infections (33.1%) is higher than a recent estimate of 18.79% based on Iranian national nosocomial infection surveillance data (11). Regional variation in HCAI prevalence could be explained by differences in geographical distribution, infection control policies, studied population, and catheterisation-related factors. Similarly, there is a similar degree of heterogeneity in reported catheter-related bloodstream infections incidence/prevalence worldwide (12-16).

Gram-positive and Gram-negative isolates occurred at nearly the same rate in this study. Gram-positive cocci, particularly staphylococci, are generally considered the most common cause of catheter-related bloodstream infections (17). However, some reports indicate that Gram-negative organisms are increasing, mainly due to their ability to acquire multidrug resistance mechanisms (12,13,18).

According to recent estimates the rate of methicillin-resistant *Staphylococcus aureus* (MRSA) infection in our study (33%) was higher than the overall prevalence (21.3%) of MRSA in a national blood sample (19). In comparison, our methicillin-resistant coagulase-negative staphylococci rate (56%) was lower than the national estimates (73.9%) (20). Despite the increasing prevalence of drug-resistant strains, several studies supporting our findings identified aminoglycosides and co-trimoxazole as potentially effective antibiotics against staphylococci clinical isolates (21-24). Our results have shown a very high rate of overall resistance and a diminishing choice of effective antibiotics, particularly for Gram-negative bacteria. This would indicate an urgent need to improve infection control and prevention of catheter-related bloodstream infections. Consistent with our findings, it seems that carbapenem resistance is increasing in the country (25-27).

In summary our study discovered a significantly high rate of intravascular catheter-associated infections, as well as an alarmingly high rate of multidrug-resistant Gram-negative organisms. This finding necessitates the implementation of a more effective infection control policy immediately. Additionally, our observations provide critical data for making more targeted empirical antibiotic selections based on the local antibiotic susceptibility pattern. Finally, it is recommended that additional studies using larger sample size and a multicentre approach be conducted to determine the factors affecting the incidence, aetiology, and trends of antibiotic resistance in catheter-related bloodstream infections.

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ORIGINAL ARTICLE

Relationship of lipid profile and erythrocyte indices in non-anaemic elderly

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ABSTRACT

Objectives: Several human studies have reported a variety of morphologic alterations in erythrocyte in relation to serum lipid components in different pathological states, but there are few reports about the influence of lipid profiles on heamatologic parameters in healthy individuals. This study evaluated the effect of elevated serum level of the lipid indices on the alteration of the RBC indices in healthy elderly persons.

Methods: 275 non-anaemic elderly people were assessed for the association between dyslipidemia and circulating erythrocyte indices in the southwest of Iran. The student t-test and Chi-square test were used for statistical comparison.

Results: There was a statistically significant difference between elderly women with and without hypercholesterolemia and hypertriglyceridemia, and in age-matched men with regard to the red cell count, haemoglobin, and haematocrit ($p<0.001$) but not for mean cell volume ($p>0.05$). Fasting levels of LDL-C were significantly associated with erythrocyte number, hematocrit, and haemoglobin concentration ($p<0.001$) in healthy elderly men and women while HDL-C levels were only associated with red cell count ($p<0.001$) and haemoglobin ($p<0.05$).

Conclusions: We conclude that dyslipidemia appears to have a significant effect on red cell indices in non-anaemic elderly persons.

Key words: Dyslipidemias; erythrocyte indices; elderly, non-anaemic.

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INTRODUCTION

Red blood cell's (RBC) plasma membrane is composed of the phospholipid bilayer, transmembrane (integral) proteins, and a cytoskeletal network of protein fibres that help to maintain the normal structure and function of the erythrocytes (1). The structural elements and biochemical composition of the RBC membrane have been well characterised (2) in that proteins constitutes approximately 52% of lipids (including cholesterol and phospholipids) 40% of carbohydrates and 8% of the mass of the RBC membrane (3). The major class of lipid composition of RBC membranes are either phospholipids or neutral lipids and unesterified cholesterol, found in about equimolar quantity (4).

The reverse cholesterol transport pathway involves the removal of cholesterol from peripheral tissue for excretion in the faeces (5). The RBC cholesterol pool is more readily exchangeable facilitating peripheral cholesterol efflux to the faeces, particularly in subjects with low HDL-c (6). Because RBCs do not contain intracellular membranous structures, a bidirectional *in vivo*, and *in vitro* efflux of free cholesterol between plasma lipoprotein and RBC plasma membranes (7) cause alterations in the fluid properties and function of the erythrocyte membrane with potential pathophysiological consequences (8). The results of one study confirmed that abnormalities in the composition of plasma lipoproteins in patients with hypercholesterolemia is associated with erythrocyte deformability and corresponding modifications in erythrocyte membrane lipid composition (9). Furthermore, another study reflects that the effects of postprandial plasma cholesterol and triglyceride (TG) levels lead to enhanced RBC aggregation accompanied by changes in RBC membrane lipid composition (10). The anaemia of severe burned patients is characterised by a decreased RBC half-life and abnormal RBC morphology (predominantly echinocytes) and is correlated with decrease in plasma lipid concentrations (5). Various studies have consistently reported a variety of morphologic alterations in erythrocyte indices in relation to serum lipid components among patients with various diseases, including sepsis-associated inflammation, (11) and chronic alcoholism (12).

A few reports have determined the influence of lipid profiles on haematologic parameters in apparently healthy individuals (5). More research is needed that provides insight into advancing our understanding of the underlying association between hyperlipidemia and erythrocyte indices in humans. We therefore investigated how elevated serum levels of the lipid panel [total cholesterol (TC), high density cholesterol (HDL-C), low density cholesterol (LDL-C) and triglycerides] alters RBC indices with a negative impact on the distribution of red blood cell mean corpuscular volume (MCV) in non-anaemic elderly individuals.

MATERIALS AND METHODS

The present-based cross-sectional study was conducted on an elderly healthy population in the southwest of Iran from September 2017 to April 2018. The Cochran formula was used for calculating of the sample size.

$n = ((z^2pq) / e^2)/1 + 1/N((z^2pq) / e^2 - 1))$ = 269

n = sample size, N = population size (900 households), z = standard normal deviation set at 95%, confidence level = 1.96, p = percentage picking a choice or response = 0.5, e = margin of error (desired level of precision) = 0.05.

Complete blood cell count (CBC), including red cell indices, were measured in 275 elderly persons, aged 50 to 65 years (121 elderly men, mean: 56.71 years; 154 elderly women, mean: 56.95 years). We excluded those taking medications for anaemia, such as for iron deficiency anaemia, chronic kidney disease, chronic inflammation, immune disorders, and smokers. This study was approved by the Ethical Committee of Abadan Faculty of Medical Sciences (IR.ABADANUMS.REC.1396.243) and informed consent was obtained from all subjects. According to the WHO definition of of anaemia, cut-off value of haemoglobin (Hb) levels of < 120 g/L was used for women and < 130 g/L for men. Serum concentrations of cholesterol, triglyceride, LDL-C, and HDL-C were measured with an automatic chemical analyser (Hitachi 747, Hitachi, Tokyo, Japan). The subjects were assigned into two groups: subjects with hypercholesterolemia (serum cholesterol ≥ 5.17 mmol/L) and with normal serum cholesterol levels of < 5.17 mmol/L. In addition, subjects were assigned

into each six groups: subjects with hypertriglyceridemia (serum triglyceride ≥ 2.26 mmol/L) and with serum triglyceride concentrations < 2.26 mmol/L; subjects with elevated serum level of LDL-C and HDL-C(serum LDL-C ≥ 2.59 mmol/L and HDL-C≥ 1.55 mmol/L and with normal serum level of LDL-C concentration < 2.59 mmol/L and HDL-C concentration < 1.55 mmol/L.

After fasting for > 12h, venous blood was drawn into an evacuated serum separator tubes. Full blood cell and haematological parameters (WBC, RBC, Hb, Hct, MVC, MCH, and MCHC) were measured with EDTA-anticoagulated blood using an electronic cell counter (SE 9000, Sysmex, Kobe, Japan). Morphologic changes in erythrocytes were examined in peripheral blood smears from all subjects. Statistical analysis was done by SPSS version 24 statistical software (SPSS Inc., Chicago, Illinois, USA). We used the student t-test for statistical comparison of groups and Chi-square test for calculating of the qualitative variables. Descriptive statistics were generated (means, or percentages and associated standard errors). P < 0.05 was considered statistically significant.

RESULTS

Descriptive characteristics of the subjects stratified by study design

The average age of participants (n=275) was 56.95± 5.35 and 56.71± 4.41 years for men and women, respectively. 121 (44%) men and 154 (56%) women had elevated serum cholesterol and triglyceride levels. Mean levels of total cholesterol and triglyceride in these men and women were 5.17 ± 1.06 mmol/L and 2.40 ± 0.53 mmol/L, respectively. Across this study, the mean (standard deviation) LDL-C level were 3.11 (0.85) mmol/L in men and 3.31 (1.03) mg/dL in women, respectively. In addition, HDL-C levels were 1.3 (0.35) mmol/L in men and 1.38 (0.30) mmol/L in women, respectively. Descriptive characteristics of the mean ±SD for haematologic parameters and red cell indices (WBC, RBC count, Hb, Hct, MCV, MCH, MCHC) are shown in Table 1.

Associations between serum cholesterol and triglyceride concentrations and erythrocyte indices in elderly men and women with and without hypercholesterolemia and hypertriglyceridemia

In case of hypercholesterolemia (serum cholesterol ≥ 5.17 mmol/L) we examined the individual association between serum cholesterol and triglyceride concentrations and erythrocyte indices. A relevant association between RBC count (p=0.011) in older men and haemoglobin (p=0.002) and haematocrit (p<0.001) in older women with high serum levels of cholesterol was found (Table 2). With regard to hypertriglyceridemia (serum triglyceride ≥ 2.26 mmol/L) significant associations between the two groups in the RBC count (p=0.005), Hb (p=0.004), and Hct (p=0.001) were found (Table4). There was no significant differences for MCV (p>0.05) in the hypercholesterolemia and hypertriglyceridemia groups (Tables 2 and 3).

Relevant association between serum LDL-C & HDL-C and erythrocyte indices in elderly men and women with normal and high levels of LDL-C and HDL-C

There was a statistically significant difference between elevated level of LDL-C (≥ 2.59 mmol/L) and RBC count (p=0.049) in older men and RBC count (p=0.002), haemoglobin (p<0.001), and haematocrit (p<0.001) in older women (Table4). Although, MCV edied not show a statistically significant difference with high serum level of LDL-C in either men (p=0.329) or women (p=0.702), respectively (Table4). HDL-C levels of ≥ 1.55 mmol/L was associated with both the RBC count (p=0.009) and Hb (p=0.009) in men and Hb (p=0.017) and MCV (p=0.013) in women, while MCV was not significantly different in men (p=0.884), (Table 5).

Table1. Descriptive parameters of study population**.**

Parameters	N	Mean	SD
TC			
<5.17 mmol/L	122	4.33	0.58
>5.17 mmol/L	153	6.17	0.80
TG			
<2.26 mmol/L	216	1.37	0.43
>2.26 mmol/L	59	2.96	1.03
LDL-C			
<2.59 mmol/L	69	2.04	0.37
>2.59 mmol/L	206	3.62	0.74
HDL-C			
<1.55 mmol/L	159	1.25	0.19
>1.55 mmol/L	59	1.79	0.31
Haematologic parameters			
WBC (10^9/l)	275	6.96	2.01
RBC (10^{12}/L)	275	5.10	0.62
Haemoglobin (g/L)	275	13.74	1.56
Haematocrit (Fraction of RBC)	275	0.40	0.04
Red cell indices			
MCV (fl)	275	78.80	7.69
MCH (pg)	275	27.07	3.17
MCHC (g/L)	275	342.9	9.2

Table 2. Red cell absolute values and erythrocyte indices in elderly with and without hypercholesterolemia. Results are mean±SD.

Characteristic	Men	Men	p-value	Women	Women	p-value
	TC < 5.17mmol/L	TC ≥ 5.17mmol/L		TC < 5.17 mmol/L	TC ≥5.17 mmol/L	
No. of cases (n)	58	63		64	90	
Age (years)	56.58±4.82	56.82±4.03		57.20±6.67	56.77±4.19	
Haematologic parameters						
WBC (10^9/l)	6.64±1.75	7.01±1.78	0.092	7.12±2.65	7.02±1.81	0.722
RBC (10^{12}/L)	5.25±0.77	5.41±0.63	0.011	4.90±0.52	4.94±0.47	0.112
Haemoglobin g/L	14.59±1,78	14.52±1.54	0.114	12.87±1.23	13.26±1.06	0.002
Hematocrit (Fraction of RBC)	0.42±0.04	0.42±0.04	0.52	0.37±0.03	0.39±0.03	<0.001
Red cell indices						
MCV (fl)	80.62±0.79	78.39±7.10	0.181	77.39±8.77	78.91±7.08	0.283
MCH (pg)	27.85±3.25	27.01±2.97	0.167	26.53±3.55	27.01±2.93	0.291
MCHC (g/L)	342±10.5	343.9±0.89	0.589	342±10.3	341.7±9	0.605

Table 3. Red cell absolute values and erythrocyte indices in elderly with and without hypertriglyceridemia. Results are mean±SD.

Characteristic	Men		p-value		Women	p-value
	TG < 2.26 mmol/L	TG ≥2.26 mmol/L		TG < 2.26 mmol/L	TG ≥2.26 mmol/ L	
No. of cases (n)	58	63		64	90	
Age (years)	56.59±4.59	57.21±3.57		57.38±5.56	55.52±4.37	
Haematologic parameters						
WBC (10^9/l)	6.66±1.69	7.56±1.96	0.001	6.89±2.31	7.64±1.64	0.282
RBC (10^{12}/L)	5.32±0.70	5.41±0.72	0.005	4.92±0.50	4.94±0.44	0.573
Haemoglobin (g/L)	14.51±1.67	14.74±1.58	0.004	12.98±1.11	13.48±1.19	0.138
Haematocrit fraction of RBC)	0.42±0.04	0.43±0.04	0.001	0.38±0.03	0.39±0.03	0.178
Red cell indices						
MCV (fl)	79.72±7.29	78.34±8.39	0.541	77.99±8.01	79.46±7.20	0.496
MCH (pg)	27.51±3.08	26.99±3.33	0.526	26.61±3.28	27.46±2.87	0.484
MCHC (g/L)	344.5±10.1	343.8±8	0.780	340.8±9.9	345.1±7.5	0.524

Table 4. Red cell absolute values and erythrocyte indices in elderly with low and high levels of LDL-C. Results are mean±SD.

Characteristic	Men		p-value		Women	p-value
	LDL-C < 2.59 mmol/L	LDL-C ≥2.59 mmol/L		LDL-C < 2.59 mmol/L	LDL-C ≥2.59 mmol/L	
No. of cases (n)	32	89		37	111	
Age (years)	55.87±4.48	57.01±4.37		57.67±7.81	56.72±4.29	
Haematologic parameters						
WBC (10^9/l)	6.55±1.89	6.93±1.72	0.879	7.07±1.94	7.06±2.26	0.764
RBC (10^{12}/L)	5.23±0.92	5.37±0.61	0.049	4.85±0.53	4.95±0.47	0.002
Haemoglobin (g/L)	14.77±2.17	14.47±1.43	0.190	12.89±1.28	13.16±1.09	<0.001
Haematocrit (fraction of RBC)	0.42±0.06	0.42±0.04	0.120	0.37±0.03	0.38±0.03	<0.001
Red cell indices						
MCV (fl)	81.46±8.56	78.74±6.99	0.329	78.24±8.46	78.34±7.65	0.702
MCH (pg)	28.18±3.58	27.14±2.92	0.285	26.81±3.40	26.83±3.14	0.586
MCHC (g/L)	345.3±12.4	344±8.6	0.940	342±10	341.8±9.4	0.372

Table 5. Red cell absolute values and erythrocyte indices in elderly with low and high levels of HDL-C. Results are mean±SD.

Characteristic	Men		p-value		Women	p-value
	HDL-C< 1.55 mmol/L	HDL-C ≥1.55 mmol/L		HDL-C < 1.55 mmol/L	HDL-C ≥1.55 mmol/L	
No. of cases (n)	32	89		37	111	
Age (years)	57.25±4.30	55.94±4.39		56.81±4.23	56.63±4.56	
Haematologic parameters						
WBC (10^9/l)	6.89±1.68	6.80±1.86	0.377	7.36±2.43	6.48±1.79	0.094
RBC (10^{12}/L)	5.35±0.62	5.46±0.48	0.069	4.95±0.52	4.90±0.35	0.454
Haemoglobin (g/L)	14.50±1.43	14.33±1.40	0.009	13.16±1.17	13.20±0.95	0.052
Haematocrit fraction of RBC)	0.42±0.04	0.42±0.03	0.009	0.38±0.03	0.39±0.02	0.017
Red cell indices						
MCV (fl)	79.02±6.83	77.47±7.42	0.884	78.24±8.30	79.26±5.98	0.013
MCH (pg)	27.27±2.86	26.43±3.22	0.685	26.85±3.35	27.03±2.60	0.035
MCHC (g/L)	344.5±8.5	340.3±12.9	0.358	342.4±9.5	340.7±8.6	0.561

DISCUSSION

Our study showed that non-anaemic elderly with hypercholesterolemia and hypertriglyceridemia had significantly higher RBC count, Hb, and Hct compared to non-anaemic elderly with normal cholesterol and triglyceride levels. Those with a high serum fasting level of LDL-C had an increase in erythrocyte number, Hct, and Hb concentration, while high HDL-C level (serum HDL cholesterol ≥ 1.55 mmol/L), was associated with a decrease in RBC count and Hb.

Conditions, such as dyslipidemia/hyperlipidemia along with plasma membrane cholesterol accumulation in erythrocytes are all modifiable risk factors for hypercholesterolemic patients (13). Clinical case studies with small number of patients have provided strong evidence that there is a probable reciprocal interaction between cholesterol loading and human population erythrokinetics and the platelet count. Experimental *in vitro* studies also demonstrated the efflux of cholesterol from RBCs for generation of serum lipoproteins because of their remarkable capacity for high cholesterol absorption (14). More human subject research is needed to provides insight into advancing our understanding of the underlying association between hyperlipidemia and erythrocyte indices, which in this study was studied. In our study, in case of hypercholesterolemia, we fully agree with Choi *et al.* (14) that there is no statistically significant variations among the mean values of red cell indices between subjects with and without hypercholesterolemia and hypertriglyceridemia. We also have shown that hyperlipidemia (hypercholesterolemia and hypertriglyceridemia) is related to RBC count, Hb, and Hct, consistent with another study (14). However, recently no differences were reported between those with and without hyperlipidemia which is different to our results regarding erythrocyte number, Hct, and Hb (14).

Population studies are generally consistent with our study in that LDL-C serum levels are positively correlated with erythrocyte membrane stability and haematocrit (8). Indeed, recent studies indicate that the management of LDL-cholesterol-lowering in hypercholesterolemic multiple sclerosis patients with statin therapy leads to improvement of the erythrocyte stability. Our data can explain that LDL-C, RBC count, and Hct are all associated. And it has already reported of reduced erythrocyte numbers in *in vivo* and in hypercholesterolemia subjects (15). Sebaaly *et al.* have also shown the clinical relevance of a strong direct relationship between increased Hb and hyperlipidemia (16).

Our results provide support that targeting triglyceride and cholesterol levels can influence RBC indexes in hypercholesterolemia and hypertriglyceridemia in the elderly The results of our study are in parallel with a previous study in which a significant relationship between abnormalities in serum triglyceride and cholesterol levels and Hb were observed in patients with moderate iron deficiency anemia (17).

To date there have been a few studies demonstrating a relationship between serum total cholesterol and triglycerides and red cell membrane lipids versus MCV in certain pathological conditions. For example, Shrivani *et al.* demonstrated plasma and red cell lipids alterations in chronic alcoholism with macrocytosis pertaining to increased MCV (17). It has also been reported that plasma levels of triglycerides and RBC cholesterol accessibility were inversely correlated with the MCV in diabetic patients with hypercholesterolemia and /or hypertriglyceridemia (18). In the study of Kim *et al.* no change in red cell MCV in relation to high plasma triglyceride levels was found while mean levels of MCV were inversely correlated with high plasma cholesterol levels (19) However, similar to that study we found no evidence of significant correlations between serum cholesterol and triglyceride concentrations with MCV in healthy elderly in men versus women. It is generally accepted that HDL particles mediate cellular cholesterol efflux through the HDL-mediated pathway of RCT(14). A previous study demonstrated that high HDL concentrations have a highly significant positive correlation related to erythrocytes and platelets count but apparently unrelated to MPV (14). It has also been observed that baseline HDL-C levels were consistently and inversely associated with erythrocyte counts. In that setting, increased erythrocyte membrane cholesterol, as an important source of free cholesterol deriving from HDL-C, can possibly impede the maturation of erythrocyte and increase haemolysis through direct effects on osmotic fragility and deformability (14).

We found no significant increases in Hct, Hb, erythrocyte count, or MCV within both males and females. Table 2 presents mean hematocrit, hemoglobin, erythrocyte count, and MCV by quartiles of the mean serum HDL-C levels. In the unadjusted model, mean erythrocyte count, hematocrit was reduced as HDL-C increased between healthy elderly men and women.

In the unadjusted model, mean erythrocyte count, haematocrit was reduced as HDL-C increased between healthy elderly men and women. In our adjusted analyses there was also a consistent relationship between HDL-C and Hb, unlike other studies which have been disparate. Furthermore, in another study an inverse relationship was found between HDL-C levels and erythrocyte numbers, similar to our finding, whereas no significant differences were observed between themes. Our data suggest that serum HDL-C is related to an abundance of measures of both erythrocytes (erythrocyte number, Hct, Hb, and MCV. Importantly, this controversy observed may be due to the type of studied populations. Our study was in the elderly while other studies have been in the general populations to have an adverse effect on red cell indices.

In conclusion, dyslipidemia appears to have an adverse effect on red cell indices, particularly for RBC count, haemoglobin, and haematocrit, but not for MCV in non-anaemic elderly persons. Furthermore, it is possible that the serum lipid profile and erythrocyte indexes causally influence each other as biomarkers with a cooperative underlying cause.

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ORIGINAL ARTICLE

Diagnostic accuracy of cardiac myosin-binding protein C for acute myocardial infarction

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ABSTRACT

Background: Acute myocardial infarction (AMI) leads to increased mortality and recurrent ischemia and should be diagnosed promptly and distinguished from other causes of chest pain. It is necessary to evaluate the accuracy of cardiac myosin-binding protein (cMyC) to identify it a useful biomarker in the early diagnosis of AMI patients.

Methods: Participants diagnosed with AMI were confirmed based on clinical findings and electrocardiography and increased cardiac troponin 1 (cTn1) levels in the Emergency Department of Ahvaz Golestan Imam Khomeini Hospitals, Iran during the 2018 year. A complete blood count, serum glucose, cTn1, blood urea nitrogen, creatinine, sodium, potassium, and serum levels of cardiac myosin-binding protein (cMyC) were measured. The patients were followed up for 24 hours.

Results: Sixty-five patients with AMI were included in the study of whom 64.6% were ST-elevation myocardial infarction. The value of AUC = 1.00 (95% CI: 1.00-1.00) indicated that the CMyBP-C marker fully and correctly identified AMI individuals. At a significance level of 5%, the P-value <0.001 indicated that the CMyBP-C marker has an excellent ability to differentiate AMI.

Conclusion: Our study showed that the cMyC biomarker was significantly higher in AMI patients at all studied times and had a high diagnostic accuracy in diagnosing patients with AMI.

Keywords: cardiovascular disease; myocardial ischemia; acute myocardial infarction; myosin-binding protein c; diagnosis.

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INTRODUCTION

Acute myocardial infarction (AMI) leads to increased mortality and recurrent ischemia and should be diagnosed promptly and distinguished from other causes of chest pain (1). About 5 million emergency room visits occur each year in the USA due to chest pain. More than 800,000 patients experience acute myocardial infarction each year, and 27% of them die (2). However, since 1970, with advances in diagnostic testing and management of these patients, the mortality rate from acute myocardial infarction has decreased (3).

Diagnosis of patients with suspected AMI should focus on clinical history, physical evaluation, electrocardiography (ECG), cardiac markers, and chest X-ray (CXR) radiography (4). Characteristics of cardiac necrosis play a vital role in diagnosing AMI in individuals suspected of having non-ST-elevation acute coronary syndrome (NSTE-ACS) (5).

Cardiac troponins (cTn) have emerged as the gold standard for the diagnosis of AMI (6). Early cTn generations were not very sensitive to detecting low concentrations in the early hours following symptoms because cTn concentrations occur approximately 16 to 18 hours after the onset of symptoms of chest pain (7,8). Latest generation, high-sensitivity cTn measurement, attempt to generate low concentrations of cTn for the diagnosis and treatment of primary NSTE-ACS (9) and early reports suggested that this measurement would lead to a faster diagnosis of AMI (10). Therefore, due to limitations in sensitivity and specificity, guidelines recommend that diagnostic tests be repeated. Although many biomarkers are released rapidly during myocardial injury, none of them exceeds cTn, which is why these biomarkers are not explicitly expressed in the heart. As a result, researchers today focus on analyses that magnify periodic changes in serum cTn concentrations to improve its low positive predictive value (PPV) in the diagnosis of AMI (11).

However, it is unclear how this strategy can offer a straightforward utility (11). If so, what percentage of magnification and absolute change in concentration can overcome analytical noise and biological differences (12). In addition, magnification of changes in parameters, such as concentration in delta, gender, age, and vendor-specific, are likely to interfere with the alternatives (13). Therefore, an ideal marker should be analogous to the profile of systolic-released proteins, such as creatine kinase, fatty acid-binding protein, and myoglobin. Still, its expression should be specific to heart tissue.

Sarcomeric protein, cardiac myosin-binding protein (cMyC), is one of these candidates, which has been detected during systemic proteomic analysis of coronary secretions of rat heart during ischemia (14). c-Myc is a thick filament protein and one of the most abundant cardiac proteins. c-Myc has three different isoforms encoded by three different genes: slow skeletal, fast skeletal, and cardiac. The latter case has a unique N-terminal and specific characteristics and demonstrates cardiac epitopes (15). Recent studies have shown that this protein is elevated in patients with AMI. In addition to its high specificity for heart tissue, this marker is more abundant than cTn and is released into the bloodstream faster during AMI (16,17). Therefore, it is necessary to evaluate the accuracy of C-Myc diagnosis to identify it as useful biomarker in the early diagnosis of AMI patients.

METHODS

Study design

This study was conducted according to the Standards for the Reporting of Diagnostic accuracy studies (STARDs) guideline (18). A retrospective diagnostic accuracy was conducted after receiving ethics approval from the Ethics Committee of Ahvaz University of Medical Sciences (AJUMS; Number: IR.AJUMS.REC.1397.488). The study was conducted by the Declaration of Helsinki for research involving human subjects.

Participants

Participants diagnosed with AMI were confirmed based on clinical findings and ECG and increased cardiac troponin 1 (cTn1) levels. The patients gave written informed consent in the Emergency Department of the Ahvaz Golestan and Imam Khomeini Hospitals, Iran, during the 2018 year. Our control group consisted of patients with any problems other than signs and symptoms related to MI and final other diagnosis.

Inclusion criteria:

- Age over 18 years.
- Patients with ST-elevation MI (STEMI) and non-ST-elevation MI (NSTEMI).
- Complaints of chest pain, chest heaviness, and sweating, or any evidence of angina equivalent.

Exclusion criteria:

- Pregnant women.
- Acute heart failure or congestive heart failure.

Test methods

After monitoring the patient, a blood sample was taken and sent to the laboratory for the measurement of complete blood count, glucose, troponin, blood urea nitrogen, creatinine, sodium, potassium, and serum levels of cMyC. The troponin and cMyC levels were measured using TOYO (Turklab A.S, Izmir, Turkey) laboratory kitsets. Plasma level of cMyBP-C were measured by sandwich ELISA using the MYBPC3 kit.

Blood samples were taken at onset, 3 hours later, 6 hours later, 9 hours later) and sent to the laboratory to determine serum levels of troponin while cMyBP-C levels were followed-up for 24 hours. In the end, the patient's final condition (re-stroke) was recorded. Age, gender, and other demographic information were recorded.

Data analysis

The standard deviation of cMyBP-C at 6 hours was equal to 780 ng/L. Also, the error rate was 200 ng/L, and the sample size was calculated to be 60 people (19).

Quantitative variables were reported as mean, standard deviation, minimum, maximum, and qualitative variables registered as number (percentage). The normality of quantitative variables was assessed using the Shapiro-Wilk test. The Chi-square test was used to examine the relationship between qualitative variables, and the Pearson correlation coefficient or its nonparametric equivalent (Spearman) was used for quantitative variables. Comparing the time to reach the maximum amount of protein C bound to myosin of the heart and troponin in patients with AMI was performed using paired t-test or nonparametric equivalent (Wilcoxon test). The duplicate size test or the T-model, or the Generalized Estimating Equations (GEE) model were used to analyse the data in a multivariate manner. Sensitivity, specificity, and the area quantified diagnostic accuracy under the receiver operating curve (AUC [95% CI]) against adjudicated AMI.

RESULTS

A total of 65 patients with AMI were included in the study. The mean age of patients was 57.03 years ± 12.25 and 61.5% of patients were male. Regarding AMI type, 64.6% were STEMI. Other patient characteristics are presented in Table 1. CMyBPC biomarker changes at zero, 3, 6, 12, and 24 hours after the referral had increasing and decreasing fluctuations (Figure 1). If in all patients the mean score of CMyBPC had a significant decreasing trend from the time of referral to 9 hours and then at 12 hours the referral increased relatively compared to 9 hours and then decreased again at 24 hours (Figure 2). Changes in the Troponin I biomarker between zero and 24 hours after referral also varied according to the onset group. If the level of Troponin I in the group of patients with pain onset time of "0–3 h" and "3–6 h" during zero to 24 hours after referral showed an ultimately increasing trend, but in the group of patients with the time of onset of pain> 6 h had an entirely decreasing trend (Figure 2).

Troponin and CMyBP-C in the AMI group were significantly higher at all studied times compared to the control group (P <0.001). The value of AUC of 1.00 (95% CI: 1.00-1.00) indicated that the CMyBP-C marker fully and correctly identified all AMI individuals (Figure 3).At a significance level of 5%, the P-value <0.001 indicated that the CMyBP-C marker has an excellent ability to diagnose disease in the patients.

Table 1. Studied variables during different periods of time in both control and case groups

Group Variables		Control (N = 60)	Case (MI patients) (N = 65)
Age, year, N (%)	18-29	5 (8.3)	1 (1.5)
	30-59	52 (86.7)	38 (58.5)
	≥ 60	3 (5)	26 (40)
Gender, Male, N (%)		32 (53.3)	40 (61.5)
Underlying disease, N (%)	Diabetes	3 (5)	1 (1.5)
	Hyperlipidemia	5 (8.3)	1 (1.5)
	Smoking	14 (23.3)	3 (4.6)
	Blood pressure	4 (6.7)	1 (1.5)
MI, N (%)	STEMI	-	42 (64.6)
	Non-STEMI	-	23 (35.4)
ECG changes, N (%)	Lower leads	-	17 (26.2)
	Side leads	-	8 (12.3)
	Right anterior leads	-	2 (3.1)
	Wide anterior leads	-	16 (24.6)
	New LBBB	-	4 (6.2)
Pain onset time (hours), N (%)	< 3	-	26 (40)
	3-6	-	34 (52.3)
	> 6	-	5 (7.7)

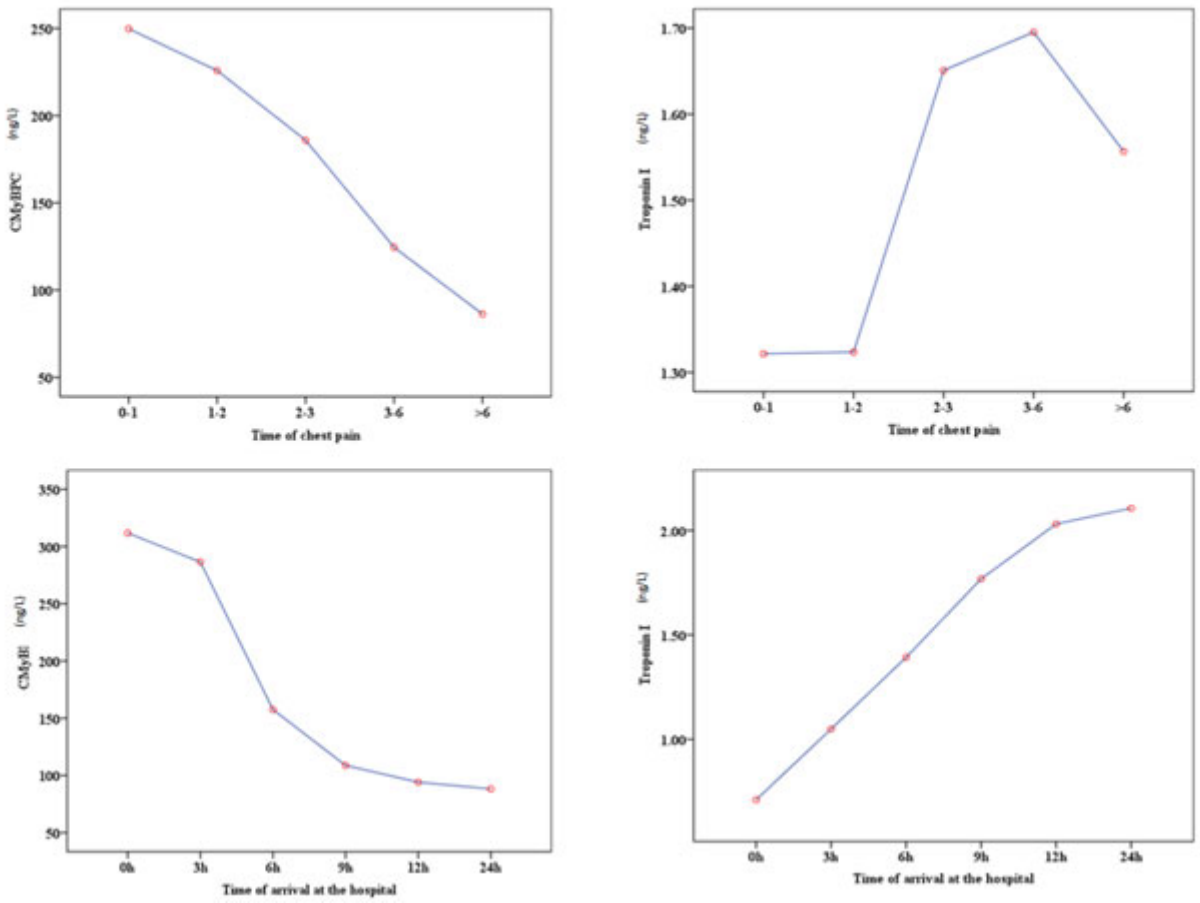

Figure 1. The trend of changes in CMyBPC and troponin levels in the patients since the onset of chest pain, and the time of arrival at the hospital.

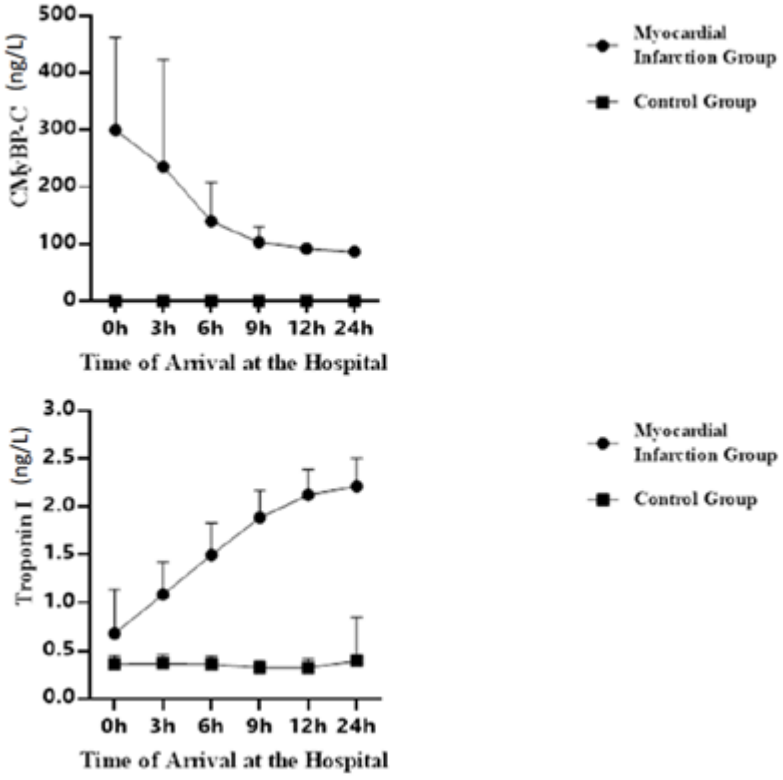

Figure 2. Trend of changes in CMyBPC and troponin at different times.

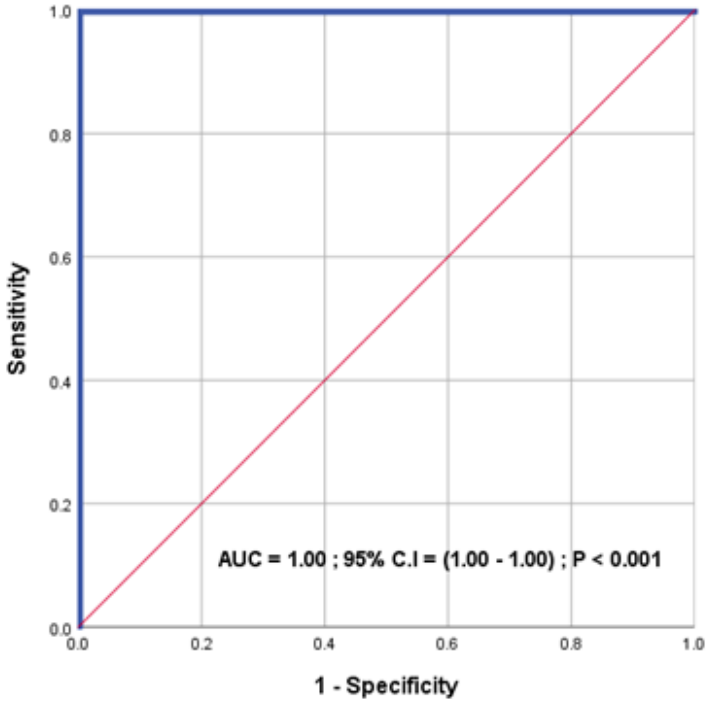

Figure 3. ROC Curve of CMyBP-C biomarker.

DISCUSSION

cMyC is the first cardiac tissue-specific protein to be reported as a diagnostic tool in AMI after cTn. In the present study, we examined changes in cMyC and cTn in AMI patients. Our research findings showed that this biomarker was significantly higher in the group of patients with AMI at all times studied and has high diagnostic accuracy in diagnosing patients with AMI. It was also found that the mean cMyC decreases as time elapses since chest pain or since hospitalisation. Therefore, the diagnostic value of this test is in the early hours.

The cMyC protein was first discovered in 1973 by Afer *et al*. with three isoforms specific for short bones, slow bones, and skeletal muscle. Skeletal muscle isoforms are widely expressed in cardiac tissue from embryonic time. Like cTnT and cTnI, this protein has a specific expression for heart tissue, but its amount is much higher (20,21).

In 2013 a study in the USA by Govindan *et al.,* calculated the trend in serum CmyBP-C levels following AMI and compared these with other available biomarkers (16). In our case-control study, serum CmyBP-C levels were measured by sandwich ELISA and CmyBP-C levels were significantly higher in AMI patients. However, a recent study has shown that CmyBP-C was 257 (75-876) ng/L for Type 1 MI with an AUC of 0.67 (95% CI: 0.61-0.73) (22). But, another study was showed high predictive power (0.967) for cMyBP-C cardiac biomarkesimilar to our results (23).

In another study biomarkers, such as myoglobin, carbonic anhydrase, and creatinine MB were significantly increased in people with MI while levels of cardiac troponin 1, glycogen phosphorylase, and cardiac fatty acids bound to the protein did not change significantly (21). The findings of that study are entirely consistent with the results of our study and showed a significant increase in CmyBP-C levels in patients with AMI. Also, it was found that the diagnostic power of CmyBP-C is high and can detect myocardial infarction with high accuracy in the early hours (21). Also, Diederik *et al.* examined the release kinetics of myosin-binding protein C in a swine model and two human groups. Measurement of CmyBP-C levels was measured 30 minutes to 14 days after coronary occlusion. The plasma level of this protein reached its baseline level (76 positive and negative 68 ng/l) three hours later (767 negative 211 ng/l) and its peak after 6 hours (2.418 positive and negative 780 ng). Per liter) was observed. The level of cMyBP-C wassimilarly reached in humans 4 hours later (12). However, in our study, it was found that the level of CmyBP-C is at its highest level in the very first hours of pain and hospitalisation and decreases over time. This difference in results may be due to sampling, differences in exclusion criteria, differences in control of the effect of confounders, differences in factors affecting CmyBP-C.

In another study patients with AMI, TASH, and CABG were evaluated (20). Serum samples were collected from these patients, and then CmyC was measured. Finally, this measurement was compared with troponin. Their results showed that following a proven myocardial injury, an increase and decrease in serum cMyC levels occurred much faster than cTnT (20). The findings of their study are similar to our study. Our study also found that CmyBP-C levels decreased over time after the onset of MI and may not be as accurate in the following hours, unlike troponin, which increased over time. Therefore, the use of CmyBP-C in the early hours has high diagnostic accuracy in diagnosing AMI.

In another study CmyBP-C levels were measured in heart failure patients at time of referral and one month after treatment. CmyBP-C levels in patients with heart failure (positive: mean 122.44 ng/ml; negative: mean 1.01 ng / ml) at the time of referral were significantly higher than the control group (positive: mean 24.40 ng/ml; negative: mean 9.83 ng/ml). The increase in CmyBP-C was associated with the severity of heart failure according to the Ross classification. In addition, the level of CmyBP-C was significantly associated with echocardiography and clinical evaluation of the heart. Finally, CmyBP-C was a promising biomarker for detecting heart failure with 100% sensitivity and 96% specificity and a 45 ng/ml cut-off (24). Also, Tong *et al.* showed CmyBP-C to be usefulness as a predictor of cardiovascular events with an AUC of 0.91 (25). That study's findings are in line with our results and show a significant increase in CmyBP-C in AMI. The AUC in our study is equal to 1, which is the highest possible level and shows CmyBP -C's high accuracy in diagnosing AMI.

Our study had sample size limitations that suggests using a widening sample size in cardiovascular disease as a predictive indicator. In conclusion, findings of our study showed that the cMyC biomarker was significantly higher in the group of patients with AMI at all studied times and had a high diagnostic accuracy in diagnosing patients with AMI. It was also found that

cMyC levels decreased as time elapses since chest pain or since hospitalisation. Therefore, the diagnostic value of this test is in the early hours. Due to the high accuracy of cMyC in diagnosing AMI, it should be used as a diagnostic test together with other tests to confirm AMI in the early hours.

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ORIGINAL ARTICLE

The Pacific Pathology Training Centre external quality assessment programme

Navin Karan, Philip Wakem, Filipo Faiga and Russell Cole

ABSTRACT

The Pacific Pathology Training Centre (PPTC) external quality assessment programme (PPTC-EQAP) commenced in 1985 as an evaluation process of students attending residential courses. This was enhanced when the PPTC was conferred Collaborating Centre status by the World Health Organization and is now the main EQA provider to the laboratories in the Pacific region.

The PPTC accommodates seven medical laboratory science disciplines within its EQA programme, and these include serology, blood bank, microbiology, haematology, biochemistry, anatomical pathology, and molecular SARS—CoV-2. Samples for each survey are dispatched from Wellington, in lyophilised form or as a whole specimen, following the IATA shipping of biological substance guidelines. All disciplines are delivered over three cycles except for biochemistry, which consists of two cycles with four analyses. The PPTC contracts consultants (registered New Zealand Medical Laboratory Scientists and a Pathologist) who are specialists in their selected disciplines for analysis and reporting of the results.

Participating laboratories are given five weeks to process the samples and return their results to the centre. Interim reports are provided a week after the due date for all programmes. A final report compiled by the appropriate PPTC consultant is sent to each participating laboratory for the specific discipline that the laboratory has participated in and each report sent details the laboratory's score for the respective cycle, their accumulated score for the previous cycle in that discipline, and the average score for all participating laboratories. This year (2021) there are 86 laboratories from 22 countries participating in all or part of the PPTC EQA programme. The New Zealand Government, through the Ministry of Foreign Affairs and Trade (NZ-MFAT), provides funding to the PPTC to deliver the EQA programme at no cost to 31 laboratories in 17 countries, while the rest of the laboratories are privately enrolled through their own funding or through donor partner funding. The PPTC provides a free external quality assurance programme for government owned and operated medical laboratories in the South Pacific region.

Keywords: external quality assessment, medical laboratory science, Pacific.

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INTRODUCTION

Assessment is a critical aspect of laboratory quality management, and external quality assessment (EQA) is one of the most commonly employed assessment tools for clinical laboratories. Proficiency testing is a process whereby an external provider sends unknown samples for testing to a set of laboratories, and the results of all laboratories are analysed, compared and reported to the participating laboratories. This is the most common type of EQA employed by medical laboratories as it efficiently addresses multiple laboratory methods (1,2).

An EQA programme provides valuable information to the participating laboratory. It allows comparison of performance and results among different test sites, provides early warning for systematic problems associated with kits or operations, provides objective evidence of testing quality, indicates areas that require improvement, and identifies training needs. EQA helps to ensure customers, such as physicians, patients and health authorities, that the laboratory can produce reliable results. Individual laboratories can use EQA to identify problems in laboratory practices, allowing for appropriate corrective action. EQA participation will help to evaluate reliability of methods, materials and equipment, as well as evaluate and monitor training impact (1).

International standards organisations recognise the importance of proficiency testing and EQA. ISO/IEC 17043:2010 quote that "*Proficiency testing schemes are inter-laboratory comparisons that are organised regularly to assess the performance of analytical laboratories and the competence of the analytical personnel*" (1,2,3). The Clinical and Laboratory Standards Institute define EQA as: "*A program in which multiple samples are periodically sent to members of a group of laboratories for analysis and/or identification; whereby each laboratory's results are compared with those of other laboratories in the group and/or with an assigned value and reported to the participating laboratories and others"* (1,2,4).

The PPTC External Quality Assessment Programme (PPTC-EQAP) commenced in 1985 as an evaluation process of students attending residential courses. This was enhanced when the PPTC was conferred collaborating centre status by the World Health Organization (WHO) in 1990. The PPTC is now the main EQA provider to medical laboratories in the Pacific region. Pacific laboratories differ considerably in the levels of instrumentation, equipment, methodology, and expertise available to them and thus the evaluations of the responses received from them must recognise these different levels of technology (1).

The major purpose of this programme is to actively assist and suggest ways in which a laboratory can improve the quality of its service to the patient. The PPTC-EQAP is provided at no cost to 31 government laboratories in the Pacific Island region, and four government laboratories in the Southeast Asian region. Funding is received from the New Zealand Ministry of Foreign Affairs & Trade (NZAID) for the provision of this programme. Furthermore, private registration of laboratories is welcomed at a nominal cost. Further support is provided to the PPTC's EQAP by the New Zealand Institute of Medical Laboratory Science (NZIMLS), Royal College of Pathologists of Australasia Quality Assurance Programs (RCPA-QAP - Sydney, Australia), Wellington Southern Community Laboratories (WSCL - Wellington Hospital, NZ), Canterbury Health Laboratories (CHL - Christchurch Hospital, NZ), New Zealand Blood Services (NZBS - Wellington and Christchurch, NZ), Whangarei Hospital, LabPLUS (Auckland Hospital, Auckland, NZ) and Institute of Environmental Science and Research – (ESR - Wellington, NZ).

MATERIALS AND METHODS

All laboratories are required to complete an EQA enrolment form for each dispatch year, providing information on the programmes they wish to enrol for, and the updated shipping and contact details for key laboratory personnel. The dispatch schedule for each year is discussed in the annual EQA consultants meeting held at the PPTC at the beginning of each year, and this schedule is shared with all participants once they have completed the enrolment form.

The PPTC EQA programme includes seven panels in the medical pathology disciplines of anatomical pathology, biochemistry, blood bank, haematology, microbiology, molecular SARS–CoV-2 and serology. Samples for each survey are dispatched from Wellington, in lyophilised form or as a whole specimen, following the IATA shipping of biological substances guidelines. All disciplines are delivered over three cycles except for Biochemistry which consists of two cycles with four analyses (1).

The PPTC EQAP is managed by the PPTC's Programme Manager, Navin Karan, through assistance from Filipo Faiga, Biochemistry Technical Specialist.

Disciplines in the PPTC EQA programme

The anatomical pathology panel consists of four to six unstained tissue biopsies mounted on microscopic slides from cases common to the Pacific region. The participating laboratories are required to perform haematoxylin and eosin (H&E) stain on the slides, and report back on target diagnosis, grade, stage of tumour/ disease, and adverse factors. The slides are prepared at Whangarei Hospital and sent to the PPTC for relabelling and preparation for dispatch. The anatomical pathology consultant is Dr Vladimir Osipov, Anatomical Pathologist, Whangarei Hospital, Northland District Health Board.

The biochemistry panel offers testing for general chemistry analytes and HbA1C. Each general chemistry panel contains four lyophilised serum samples. The first lot of the samples are to be analysed immediately on receipt and the second lot of samples are to be analysed one month later. The HbA1C panel includes three lyophilised whole blood samples. The samples used for this panel are donated by the RCPA-QAP. The biochemistry consultant is Mr Filipo Faiga, PPTC Biochemistry Technical Specialist.

The haematology panel consists of three stained blood films. Participating laboratories are required to perform a differential count as well as identify and interpret WBC, RBC and platelet population abnormalities with the suggestion of a most probable diagnosis where possible. From time to time, blood films are replaced by a selection of photomicrographs from which participants will be required to answer a series of questions relating to the cell populations presented. Patient samples are obtained from the WSCL and LabPLUS, Auckland Hospital, and staining and preparation of slides are performed at the PPTC. The haematology consultants are Mr Philip Wakem, PPTC CEO and Haematology Specialist and Ms Elizabeth Tough, Haematology Specialist/Senior Morphologist and retired senior Medical Laboratory Scientist.

The microbiology panel consists of three pathogenic organisms for identification and antimicrobial susceptibility testing. A range of both Gram-positive and Gram-negative organisms are included and on occasion a more fastidious organism is included. Cultures are either lyophilised or sent on solid media. A parasitology case study with pictogram is included with each survey. On occasion mixed cultures, samples requiring urine cell count, and Gram stains may be included as sample types. The bacterial cultures are prepared at the microbiology laboratory, WSCL or are donated by the RCPA-QAP. The PPTC prepares and performs in-house lyophilisation of cultures in multiple transportation vials, and these are prepared for dispatch and testing by the participating laboratories. Consultant for the Microbiology programme is Ms Nicky Beamish, senior Medical Scientist, WSCL.

The molecular SARS–CoV-2 panel consists of three heat deactivated nasopharyngeal samples in VTM from anonymised potential positive COVID-19 patients, supplied by ESR-NZ. Each sample has a reproducible Ct value within a range of moderate to weak positive Ct values. The QC material must go through an extraction process, similar to patient samples. Samples are suitable to use with open RT PCR platforms and with the other point of care RT-PCR technology, such as Gene Xpert/ Biofire analysers. Stability testing is carried out by ESR for each sample prior to dispatch. Samples used for the molecular COVID-19 rounds are also sent to 28 IS015189 accredited laboratories in New Zealand as part of the ESR's New Zealand SARS-CoV-2 EQAP. Results generated from the Pacific cohort are compared to those from New Zealand accredited laboratories for validation purposes only. All results from the participants are shared with ESR for marking and report generation.

The transfusion science/ blood bank panel consists of blood and plasma from a 'recipient' and three samples of red cells from possible 'donors'. Blood products (donated by the NZBS) are used in the preparation of the samples. The participants are required to carry out blood grouping (ABO and Rh), antibody screening on each sample, and cross match the donor's samples with those of the patients. From time-to-time antibodies are included to ensure some donors are incompatible. Initial sample selection is carried out at the Wellington blood bank, which are then aliquoted into smaller volumes and prepared for dispatch and testing. The blood bank consultant is Mr Dan Gyles, Team Leader and Medical Scientist, Wellington blood bank, NZBS.

The serology or infectious diseases panel consists of seven serum samples per cycle. Target infectious diseases include HIV, Hepatitis B, Hepatitis C, Syphilis and Dengue. Expired plasma blood products (donated by NZBS) are spiked with patient samples positive for the target agent. These are prepared at CHL and sent in bulk to the PPTC where they are aliquoted into smaller volumes and prepared for dispatch and testing by the participating laboratories. The PPTC Consultant for the Serology programme is Ms Donna Mitchell, senior Medical Scientist, CHL.

Participating laboratories are given five weeks to process the samples and return their results to the centre. Results are submitted to the PPTC via email on a respective worksheet provided for each discipline. Sample selection, bulk preparation, testing and development of referee reports, and formalisation of the final reports are carried out by individual PPTC contracted consultants (senior registered New Zealand medical laboratory scientists and a pathologist) who are specialists in their selected discipline. A referee's report (reference results are developed from test results reported by ISO 15189 accredited laboratories in New Zealand. For biochemistry, RCPA-QAP validated results are used) is provided with the correct answers to all laboratories enrolled in each of the respective disciplines immediately after the due date to encourage corrective action. Results received from each participant are evaluated by the appropriate PPTC consultant, and a final report is prepared. Laboratories who have not participated are encouraged to use the referees report to undertake corrective action. Each laboratory is identified by a unique code number and their generated results are kept confidential by the PPTC, the WHO regional adviser of health laboratories at the regional office in Manila, and the relevant department at the NZ Ministry of Foreign Affairs and Trade in Wellington.

RESULTS

This year (2021), there are 86 laboratories from 22 countries participating in all or part of the PPTC EQA programme. The New Zealand Government, through the Ministry of Foreign Affairs and Trade (NZ-MFAT), provides funding to the PPTC to deliver the EQA programme at no cost to 31 government owned and operated laboratories in 17 countries, while the rest of the laboratories are privately enrolled through their own funding or through donor partner funding (1,6,7).

Table 1. Laboratories per country along with their appropriate funding sources for EQA.

Country	Number of laboratories	Funding source
South and North Pacific Region		
American Samoa	1	MFAT
Cook Islands	1	MFAT
Federated States of Micronesia	4	MFAT
Fiji	9	4 MFAT, 5 Self-funded
Kiribati	2	MFAT
Marshall Islands	2	MFAT
Nauru	1	MFAT
Niue	1	MFAT
Palau	1	MFAT
Papua New Guinea	2	1 MFAT, 1 Self-funded
Samoa	2	MFAT
Solomon Islands	3	MFAT
Tonga	2	MFAT
Tuvalu	1	MFAT
Vanuatu	3	2 MFAT, 1 Self-funded
Wallis and Futuna	1	MFAT
Tokelau	1	MFAT
Asia Pacific Region		
Bhutan	3	1 PPTC, 2 Self-funded
Cambodia	40	Self-funded
Laos	3	PPTC
Maldives	1	Self-funded
Timor Leste	2	PPTC

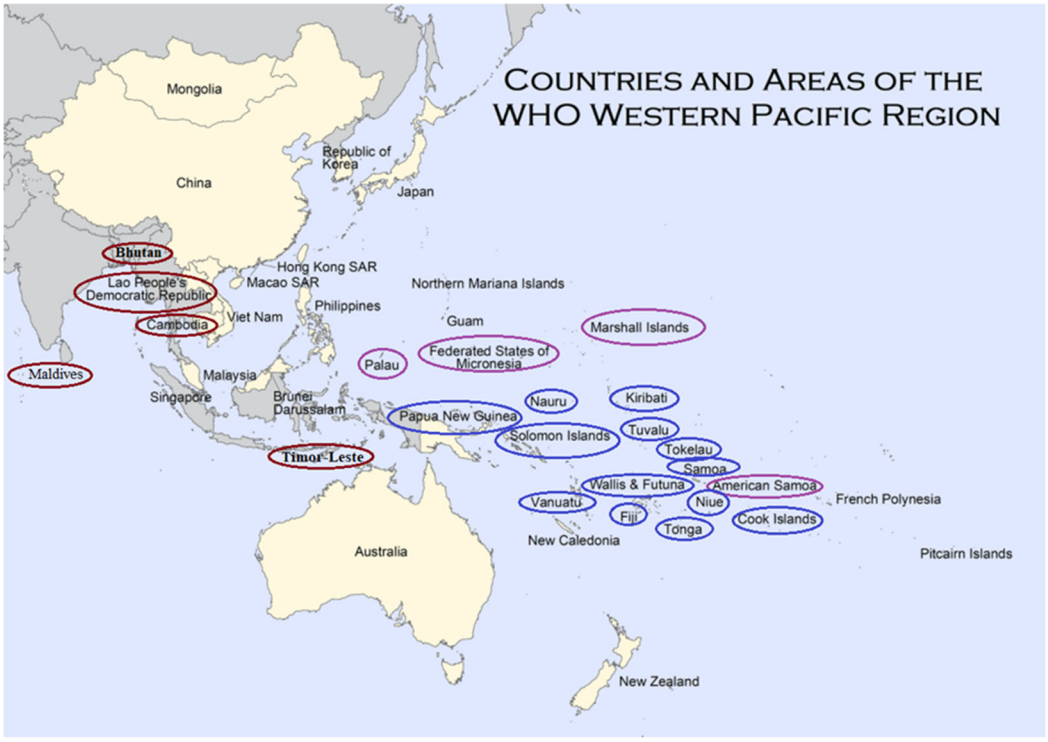


Figure 1. Geographical distribution (WHO Western Pacific Region map, 2009) of Asia-Pacific nations registered on the PPTC REQAP.

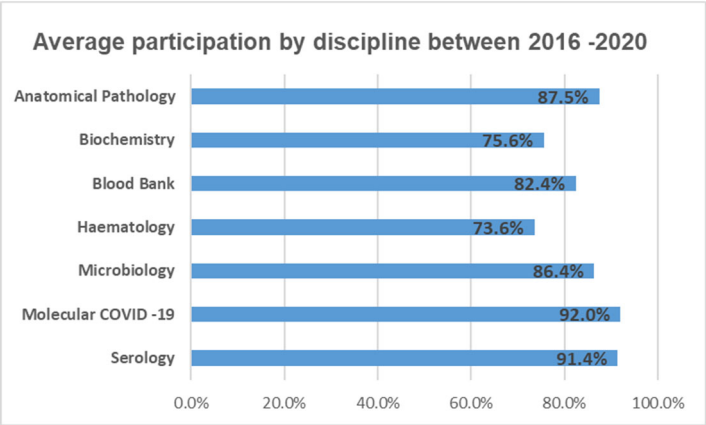
Participation

Most laboratories who enrol in the PPTC EQA programme recognise the importance of participating in it. As the Pacific countries progress in the implementation of ISO 15189 standard, there has been a greater awareness of the EQA programme in recent years. The following tables and graphs demonstrate the participation rates and performance rates of all participants in the Pacific region over the last five years. The results displayed represent all laboratories registered in the PPTC's REQA programme including those funded by the New Zealand Government and supported by the PPTC (1,6,7).

Table 2. Participation achieved in each discipline per year (2016-2020).

Participation rates – 2016-2020					
	2016	2017	2018	2019	2020
Anatomical pathology	-	-	-	94%	81%
Biochemistry	70%	67%	88%	86%	67%
Blood bank	79%	84%	90%	87%	72%
Haematology	75%	73%	76%	81%	63%
Microbiology	82%	82%	91%	93%	84%
Molecular COVID-19	-	-	-	-	92%
Serology	84%	95%	93%	91%	94%

The above table indicates an 11% (across discipline average 78%) improvement in participation when comparing 2016 (78%) to 2019 (89%). Participation dropped down to 10% in 2020 (79%) from 2019 (89%) due to the challenges brought by COVID-19 (6, 7).



Graph 1. Five-year average participation by discipline between 2016 -2020.

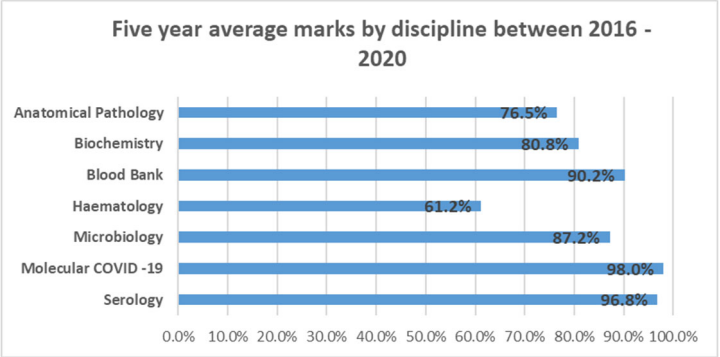
The average participation is now sitting at 84% average. The PPTC continues to email constant reminders to the laboratories to participate. Some of the reasons cited by non-participating laboratories include the availability of reagents, analyser malfunction/ breakdown and human resource issues.

Performance

Variation of marks achieved over the five years across all laboratories shows little significance except for haematology (Table 3 and Graph 2).

Table 3. Marks achieved by discipline per year (2016-2020).

Performance rates – 2016-2020					
	2016	2017	2018	2019	2020
Anatomical pathology	-	-	-	77%	76%
Biochemistry	81%	79%	81%	75%	88%
Blood bank	91%	93%	93%	91%	83%
Haematology	75%	65%	55%	55%	56%
Microbiology	88%	88%	88%	87%	85%
Molecular COVID -19	-	-	-	-	98%
Serology	96%	98%	97%	97%	96%



Graph 2. Five-year average mark achieved by discipline between 2016 -2020.

Where performance is concerned, average data over the past five years indicate that all laboratories tend to perform well in molecular (98%) followed by serology (97%), blood bank (90%), microbiology (87%), biochemistry (81%), while anatomical pathology (77%) and haematology (61%) display poor performances (6,7).

Performance in haematology

Haematology continues to be weak in performance and this is due to a devastating lack of expertise and interpretative skill in blood film examination and interpretation throughout the Pacific. The PPTC offers a six-week training course each year at its centre in Wellington but can educate students only to a certain level given such a limited time frame. Blood cell identification and interpretation is a continual learning process and unfortunately Pacific Island laboratories do not have resident experts who are able to mentor and add to this learning experience (6,7).

A comprehensive knowledge (theory and practice) of both normal haematology and pathological haematology is a building process and excellence in proficiency can take several years to achieve. The PPTC recognises that long term New Zealand consultancy attachments in the Pacific would be of enormous benefit but would require extensive financial resource which unfortunately, taking into consideration PPTC's current budget, is not a reality at this time. It is hoped that in the future, a PPTC facilitated haematology strengthening programme could be developed for the Pacific, which would enable New Zealand consultants to be attached for up to six months to Pacific laboratories where comprehensive teaching and training could be offered, and skills development could be made in blood film interpretation and diagnosis (6,7).

To continue haematology strengthening in the Pacific within the realms of its financial capability, the PPTC, in addition to its on-site training commitment, introduced to Pacific Laboratories in 2018 a change in the assessment of haematology EQA. The marking schedule was reviewed by the PPTC resulting in higher expectations in terms of excellence in laboratory practice. The marking criteria is more critical and less accommodating when compared to previous years and aligned more closely to current international standards of reporting. This can be challenging, as reflected in haematology average scores for both 2018 and 2019, but with PPTC's guidance and support, laboratories accept the challenge and strive for higher quality in the results that are presented (6,7). Furthermore, since 2020 the PPTC's Haematology Specialist provides a regular remote online teaching session for the Pacific Island laboratories on morphology and cell population recognition.

Performance in anatomical pathology

There is an overwhelming deficiency of specialist and general pathologists in the Pacific region, and this has been an unresolved issue for many years. Junior pathologists who are employed in Pacific laboratories often do not have the support or ongoing mentorship essential for their individual professional development and this is reflected in their lack of cellular recognition, identification of disease patterns, and interpretative expertise. This can result in prolonged illness, misdiagnosis, and mismanagement of treatment (6,7).

In 2021, the PPTC is introducing a Motic automatic slide scanner with tele-pathology capability in Tonga, Solomon Islands, and Vanuatu. The PPTC is also working with pathologists in New Zealand to form a functional pathology working group to provide a tele-referral service to these countries. This will develop in an extensive teaching and upskilling opportunity for the junior pathologists currently working the Pacific region.

Performance in biochemistry

For biochemistry there can be a general lack of equipment maintenance (including annual preventative maintenance programmes) and assay reagents are not regularly checked and calibration that is often overlooked. Moreover, insufficient operator and troubleshooting training is provided to staff which further compromises assay performances and patient results. Regular review of the internal quality control is encouraged through the EQA programme (6,7).

To address the deficiencies in biochemistry, the PPTC's Biochemistry Technical Specialist provides remote online training workshops addressing quality control, calibration, and troubleshooting guidelines. When approached by the countries the PPTC provides analyser selection advice as well to ensure the most appropriate analysers are purchased by laboratories.

Performance in microbiology

In microbiology organism identification and antimicrobial susceptibility testing (AST) interpretation can be challenging for some laboratories. Limitations in AST is the lack of knowledge and understanding of intrinsic resistance versus in vitro test results, the use of correct disc concentrations, and the application of disc diffusion versus MIC testing. As with other programmes the use of in-date reagents and consumables and performing regular internal quality control is encouraged (6,7).

Performance in blood bank and serology

In blood bank and serology expired kits may be infrequently used and internal quality control on occasion is overlooked. The participating laboratories are encouraged to implement an expired kit/reagent policy in the laboratory stating that routine testing should not be performed on expired reagents/kits. A procurement strategy to ensure in-date kits are available for routine testing is also recommended (6,7).

Performance in molecular COVID-19 testing

Both participation rates and performance rates overall can be considered to be excellent since the programme's implementation in 2020. Lack of participation or a delay in returning results, if it does occur, can usually be attributed to delays or failure in the delivery of samples. This can be caused by long transit times and courier disruptions as a direct result of the current pandemic (6,7).

At the end of each year, participating laboratories are provided with individualised, discipline specific annual reports with recommendations for improvement. A summary report is also presented to the Ministry of Health and hospital administrators for their information (1).

CONCLUSIONS

Through the financial support of the New Zealand Government, and support from the NZIMLS, RCPA-QAP, WSCL - Wellington Hospital, CHL - Christchurch Hospital, NZBS - Wellington and Christchurch, Whangarei Hospital, LabPLUS - Auckland Hospital, and ESR, Wellington the PPTC has been able to provide a free external quality assurance programme for government operated medical laboratories in the South Pacific region. As the Pacific countries progress in the implementation of ISO 15189 standards and work their way towards accreditation, awareness has improved in recent years in the importance of participating in a proficiency testing programme. Furthermore, performances have improved in all disciplines as quality standards are improved. It is hoped that participation and performances in the poor performing disciplines will also improve as the PPTC continues to offer professional development opportunities for the Asia-Pacific region through its distance taught Diploma programme, discipline specific centre based (Wellington) short term courses, remote online teaching, and through in country training visits.

ACKNOWLEDGEMENTS

The PPTC would like to take this opportunity to thank all the participating laboratories who continue to seek excellence in their performances to improve patient outcomes, the donor agencies, supporters, and friends of the PPTC in the provision of this programme to the developing nations.

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CASE STUDY

An anastomosing haemangioma within a lymph node: a rare vascular tumour in a novel location

Yee Sing Lin, Andrew Parasyn and Trent Davidson

ABSTRACT

Anastomosing haemangioma is a rare, benign vascular tumour with a predilection for genitourinary and paravertebral sites, but which has been described in a number of other locations. We report a case of an anastomosing haemangioma in a lymph node presenting as a painful right axillary mass. Microscopic examination revealed a characteristic non-lobular proliferation of anastomosing, thin-walled capillaries, with focal endothelial hob-nailing, and the absence of significant cytologic atypia or mitotic activity.

Keywords: anastomosing haemangioma, lymph node.

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INTRODUCTION

Anastomosing haemangioma is a rare, benign vascular tumour first described in the genitourinary tract (1). Histologically, the lesion is characterised by anastomosing sinusoidal capillary sized vessels with occasional hobnailed endothelial cells, fibrin thrombi, and absence of mitotic activity and cytologic atypia (1) and can be easily misinterpreted as a well differentiated angiosarcoma. This lesion has since been noted in the genitourinary tract (1), para-vertebral soft tissues (2), gastrointestinal tract (3), skin (4), gynaecologic tract (5), upper respiratory airway (6,7), and a variety of other viscera (8-10).

We report a case of an anastomosing haemangioma identified within a lymph node and presenting as a painful right axillary mass.

CASE

A 48-year-old female presented with a tender mass in the right axilla. The initial clinical diagnosis was of a neuroma. MRI revealed a round enhancing lesion with a possible hilum. After resection, a 10mm lymph node was received with multiple attached fragments of fat. Microscopically there was a 7mm benign vascular proliferation expanding and occupying the vascular sinusoid of a compressed lymph node. The lesion was largely circumscribed; however, did not have a lobular architecture, and consisted of anastomosing, variably dilated, thin walled vessels lined by endothelial cells with focally hobnailed nuclei.

Centrally, there were thick walled arteriolar type vessels. There was no endothelial atypia, multilayering, or mitotic activity. The stroma between the vascular lumina contained plump spindled cells and appeared hyalinised and a 3.3mm central portion of the lesion showing marked oedema of the stroma, possibly representing secondary trauma related change. Occasional mast cells were present within the tumour. CD31 and CD34 highlighted the vascular endothelial cells while the intervening stromal cells were negative. D2-40 did not stain the tumour cells, however, highlighted subcapsular lymphatic sinuses. SMA was positive in surrounding pericytic stromal cells.

The associated lymphoid tissue appeared attenuated and reactive. No abnormal lymphoid population was identified on flow cytometry. The morphologic appearances of the lesion were felt to represent an anastomosing haemangioma arising within a lymph node. Three additional reactive appearing lymph nodes were also seen in the submitted fat. These were uninvolved by the anastomosing haemangioma.

DISCUSSION

Lymph nodes are a distinctly rare site for vascular tumours. The morphologic spectrum of such tumours includes haemangiomas, angiomyomatous hamartomas, epithelioid vascular tumours (such as epithelioid haemangiomas, epithelioid haemangioendotheliomas, and epithelioid angiosarcomas), polymorphous haemangioendothelioma (a rare, vascular tumour composed of variable solid, primitive, and angiomatous components with borderline malignant potential), lymphatic malformations, and Kaposi's sarcoma (11). Intranodal papillary intravascular angioendotheliomas, also known as Dabska tumours, have also been described (12).

Anastomosing haemangiomas are rare and were first described in the genitourinary tract (1). They are benign and have been noted in the kidney, testis (1), mediastinum, uterus, para-aortic and para-vertebral soft tissues (2), adrenal (10, liver, small bowel, colon (3), skin (4), breast (9), ovary (5), larynx (6), nasal cavity (7), and atrium (8). They can occasionally be multifocal.

The histologic features of this tumour characteristically include a non-lobular proliferation of tightly packed and anastomosing capillary-like vessels. In addition, there may be fibrin thrombi, hobnailed endothelial cells, intracytoplasmic eosinophilic globules, and adjacent extramedullary haematopoiesis. Given the non-lobular growth and occasional prominent endothelial cells, the main diagnostic differential is of a well differentiated angiosarcoma. Notably, however, there is no multilayering of the endothelial lining, high grade cytologic atypia, or mitotic activity.

In the present case we did not find eosinophilic globules within the tumour. We also noted occasional mast cells throughout the lesion, which have not been reported in anastomosing haemangiomas in other locations. Consistent with cases reported in other locations (1,3), the tumour stained positive for CD31 and CD34. Negative D2-40 staining excluded a tumour of lymphatic lineage. Fresh tissue was split for routine flow cytometry as the tumour was an unexpected pathologic finding; no abnormal lymphoid population was identified.

Anastomosing haemangiomas have been found to have recurrent mutations in GNAQ and its homologues GNA11 and GNA14 (13), suggesting it is a neoplastic, rather than a reactive process. Activating mutations in GNAQ have also been identified in other benign vascular tumours, particularly congenital vascular lesions associated with Sturge-Weber syndrome (13). However, they have not been reported in angiosarcoma suggesting that the two are not genetically related.

In summary, this is a rare case of anastomosing haemangioma reported in a lymph node, further adding to the documented anatomical extent of this tumour. Awareness of this lesion is important to avoid misinterpretation as a malignancy.

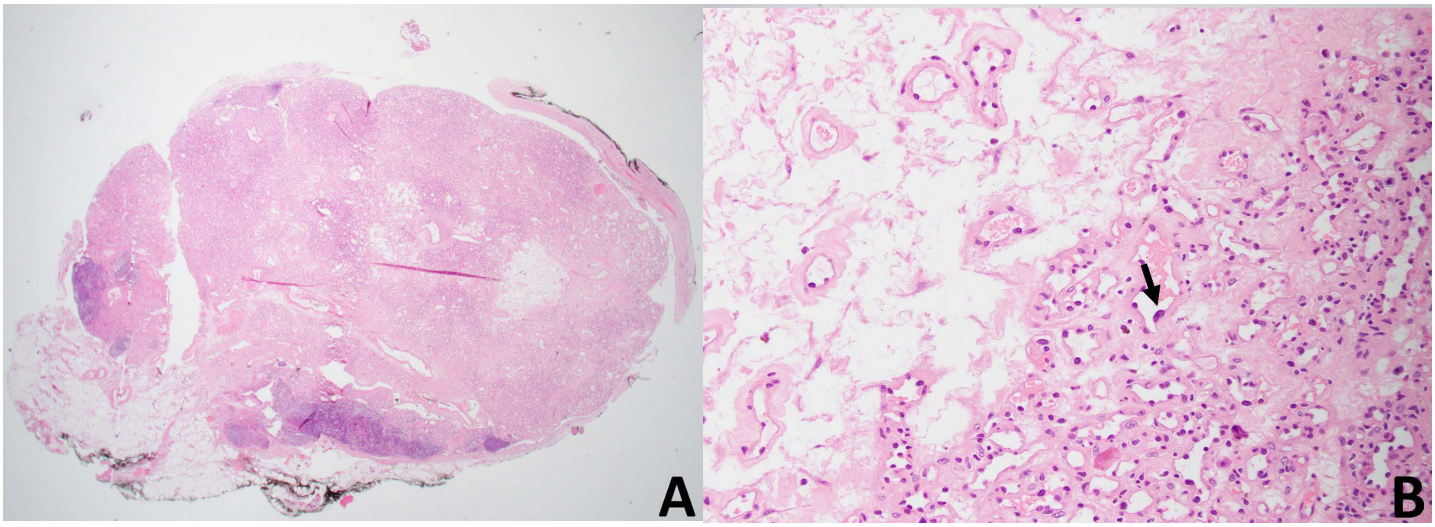

Figure 1. (A) Scanning magnification view of lymph node reveals replacement by a benign vascular lesion. Residual lymphoid tissue is compressed at the periphery. Magnification: 12.5x. **(B)** The central portion of the lesion has a significantly oedematous stroma with hyalinised vessels. Note the hobnailing of the endothelial cells (black arrow). Magnification: 200x.

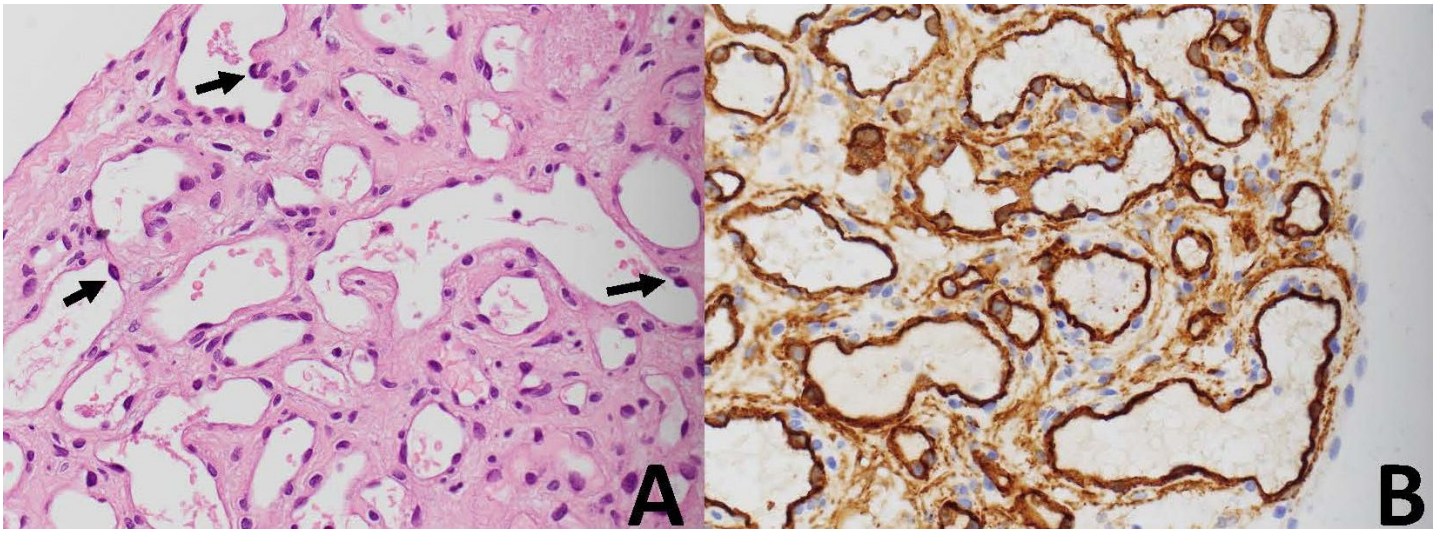

Figure 2. (A) The lesion is composed of anastomosing capillary like vessels without cytologic atypia or multilayering. Note the hobnailing of the endothelial cells (black arrow). Magnification: 400x. **(B)** CD34 stains the endothelial cells and highlights the vessels. Magnification: 400x.

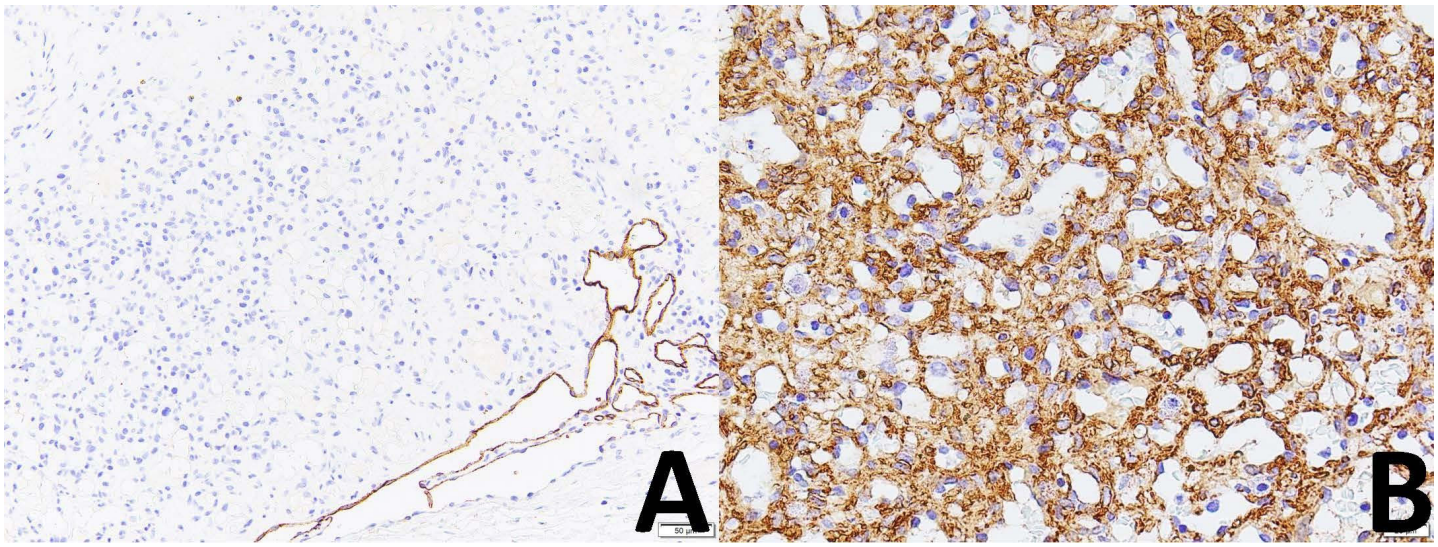

Figure 3. (A) D2-40 staining is negative in the tumour cells. The lymphatic sinuses are highlighted in the subcapsular region. Magnification: 200x. **(B)** SMA highlights the surrounding pericytic layer. Magnification: 400x.

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CASE STUDY

Chronic myeloid leukaemia presenting in blast phase - a case study

Jennie Marks

ABSTRACT

Chronic myeloid leukaemia (CML), *BCR-ABL1* positive is a myeloproliferative neoplasm characterised by a chromosomal translocation t(9;22), the Philadelphia (Ph) chromosome resulting in the *BCR-ABL1* fusion gene which manifests as uncontrolled proliferation of myeloid cells. CML is a triphasic neoplasm with a chronic phase (CP), an accelerated phase (AP), and a blast phase (BP). The majority of patients present in chronic phase. Treatment of CML in chronic phase is extremely successful with patients having a life expectancy of that of the general population. Presentation of CML in accelerated or blast phase is rare and occurs in approximately 5% of cases. We report a rare case of CML presenting in blast phase with over 20% blasts found in the peripheral blood and bone marrow.

Keywords: chronic myeloid leukaemia; blast phase, WDF scattergram

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INTRODUCTION

Chronic myeloid leukaemia (CML), *BCR-ABL1* positive is a myeloproliferative neoplasm characterised by the reciprocal translocation of chromosomes 9 and 22. This translocation results in the *BCR* gene found on chromosome 22 fusing with the *ABL1* gene found on chromosome 9 and the formation of the Philadelphia (Ph) chromosome containing the *BCR-ABL1* fusion gene (1). The resultant *BCR-ABL1* gene has dysregulated tyrosine kinase activity that causes uncontrolled proliferation of myeloid cells (2). CML follows a triphasic course with an initial indolent chronic phase followed by a more aggressive accelerated phase and blast phase (1).

Most patients, approximately 95% of cases, present in chronic phase while presentation in the accelerated or blast phase is rare (3,4). Patients with CML may present with fatigue, weight loss, fever, night sweats, abdominal fullness, signs and symptoms of anaemia, infection, or bleeding (5). Splenomegaly is present in up to 50% of cases (6). However, nearly half of patients diagnosed with CML-CP are asymptomatic and the diagnosis is made from routine blood work (6).

The complete blood count in patients with CML typically shows a leucocytosis with neutrophil and myelocyte peaks and an accompanying eosinophilia and basophilia. The presence of either leucocytosis, neutrophilia, immature granulocytes, eosinophilia, or basophilia should all trigger blood film review depending on user defined ranges set by the laboratory (7). The presence of immature granulocytes is detected by haematology automated analysers and depending on the technology used will either generate a suspect flag or enumerate the immature granulocytes. Sysmex XN automated analysers (Sysmex Corporation, Japan) use fluorescence flow cytometry principles to classify and quantify white blood cells, including immature granulocytes, according to their size, structure, and cellular complexity. The cell volume is indicated by the intensity of the forward scattered light, the internal cell structure and content is indicated by the side scatter and the amount of nucleic acids present in the cell are indicated by the intensity of the side fluorescence, which are all then expressed in a graph known as a scattergram (8) as shown in Figure 1.

The diagnosis of CML requires the detection of the Philadelphia chromosome and/or the *BCR-ABL1* gene rearrangement by molecular techniques (1). According to the WHO classification in CML-CP there are usually less than 2% blasts in the peripheral blood, in accelerated phase 10-19%, and blast phase ≥20% (1). Tyrosine kinase inhibitors (TKIs) are used for the treatment of CML and act by inhibiting the *BCR-ABL1* fusion gene (9). The use of TKI's has dramatically reduced the number of cases that progress from chronic phase to accelerated or blast phase disease (4). Patients with CML-CP that are treated with TKIs respond extremely well to treatment and have a near normal life expectancy (10), whereas patients with CML-BP often have an extremely poor outcome (11). In this case report we present a novel case of CML presenting in blast crisis with an atypical presentation and response to treatment.

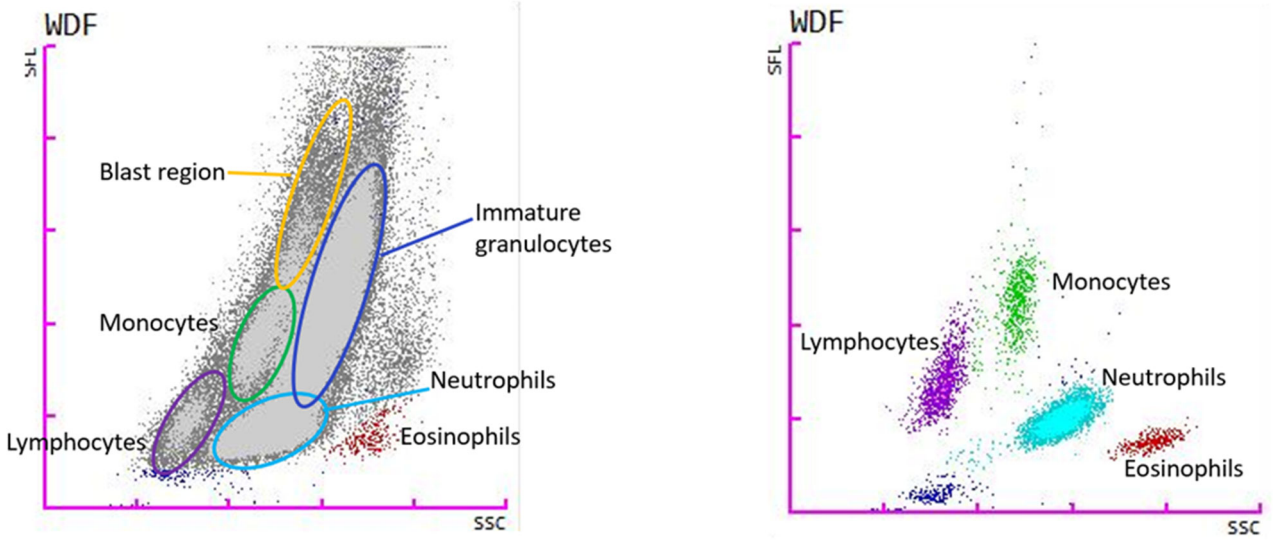

Figure 1. An example of the patient's white cell differential scattergram (WDF) on the left showing the region where the immature granulocytes are found (dark blue), one of the regions where blasts are found (yellow) and eosinophils identified shown in red. A normal white cell differential scattergram is shown on the right for comparison.

CASE REPORT

A 47-year-old male presented to the emergency department with chest pain. He had a two-day history of increasing left rib pain/chest pain on a background of heart disease with hypertension and previous ST-elevation myocardial infarction (STEMI). He denied any fevers or hot/cold flushes but did describe night sweats in the preceding six months with some unintentional weight loss noted. No bleeding, evidence of infection, headache, or blurred vision were present. On examination he was found to have no lymphadenopathy but an enlarged spleen (4 finger breadths below the costal margin) with no tenderness.

The patient's CBC revealed a markedly high WBC of 252.64 x 10^9/L, haemoglobin 132 g/L, RBC 4.95 x 10^{12}/L, and platelets of 262 x 10^9/L. Early information could be derived from the white cell differential scattergram generated by the analyser. The scattergram in this case showed a heterogeneous population with a large number of cells sitting in the immature granulocyte region and blast region found above the monocytes (Figure 1). A clear eosinophilia can also be seen. The lack of distinct separation between cell clusters caused the analyser to 'grey out' the usually coloured scattergram as it was unable to clearly classify the cells, resulting in an abnormal scattergram flag.

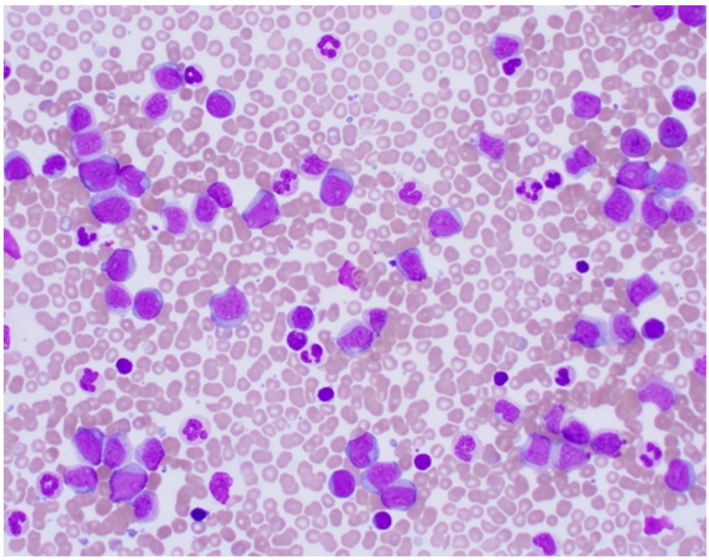

Figure 2. Peripheral blood smear at x50 magnification with many blast cells present.

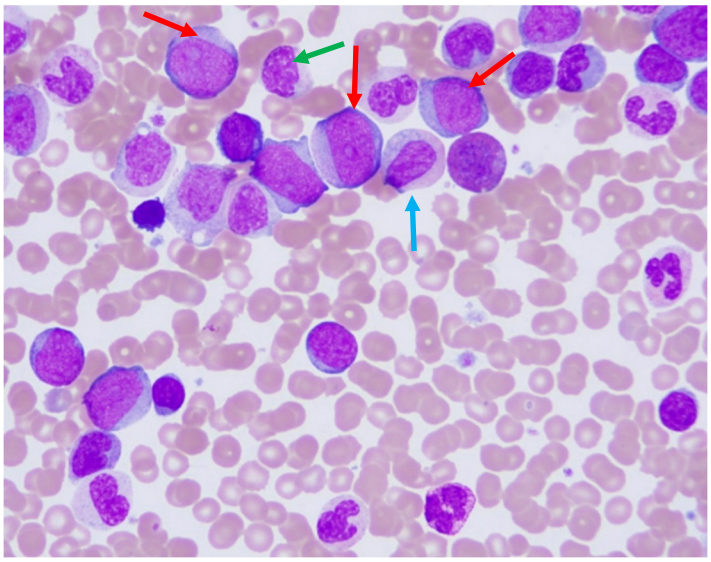

Figure 3. Peripheral blood smear at x100 magnification. Red arrows indicating blasts, blue arrows myelocytes and green arrow a metamyelocyte.

The blood film confirmed the presence of a marked leucocytosis with many immature granulocytes as well as a large number of blast cells (Figures 2 and 3). Blasts cells were large with 1-2 large nucleoli and deeply basophilic cytoplasm. No Auer rods were present. There was a myelocyte and neutrophil peak with an associated eosinophilia and basophilia.

The features seen on the blood film were consistent with CML; however, a bone marrow was required to confirm blast phase disease given the high number of blast cells present in the peripheral blood (26% of total white cells). Cytogenetic testing was performed on the peripheral blood which revealed the presence of the *BCR-ABL1* gene rearrangement, confirming the diagnosis of CML. The bone marrow aspirate mirrored the peripheral blood findings. A marked eosinophilia was noted; however, basophils were significantly less prominent. Blast cells were increased at 23% of nucleated cells. A diagnosis of CML-BP was made.

DISCUSSION

The hallmark laboratory features of CML are an increased WBC and immature granulocytes with a peak in myelocytes and neutrophils. There is often an associated eosinophilia and basophilia (1). Laboratory scientists are trained to recognise these features as potential flags for the disease. Blood film review should be triggered by these CBC findings. User defined ranges for blood film review for all CBC parameters are established by each laboratory in accordance with ISCH guidelines and clinical correlation with laboratory haematologists (7). In our laboratory we use a cut off of > 3% immature granulocytes for blood film review.

The patient in this case had a history of heart disease with previous CBCs taken at the time of each acute presentation. There is some overlap with CML, and a reactive picture caused by infection and inflammation which has the potential to make identification more difficult. A leukaemoid reaction with a marked leucocytosis is a haematological abnormality that may be confused with leukaemia (12). Features that suggest a leukaemoid reaction include toxic changes such as granulation, Dohle bodies and vacuolation due to infection, and a predominance of more mature myeloid cells. Toxic features are usually absent in patients with CML (12). The presence of a persistent thrombocytosis with large or giant platelets may support a diagnosis of CML (13). The WBC in patients with CML can range anywhere from 12-1000 x10^9/L (1,5). The platelet count is either normal or increased and in chronic phase marked thrombocytopenia is uncommon (1,5). CML-chronic phase is associated with a hypercellular bone marrow with leucocytosis with an increase in myelocyte and neutrophil fractions. There is no significant dysplasia seen (1). Blasts are usually <5 % in the marrow and <2 % in the blood. Like the peripheral blood eosinophils and basophils are increased. In the bone marrow megakaryocytes are smaller than normal and have hyposegmented nuclei. These are not true micromegakaryocytes like those seen in myelodysplastic syndromes called dwarf megakaryocytes (1).

According to the WHO criteria accelerated phase disease is defined by one or more of the following criteria: 10-19 % blasts in the peripheral blood and/or bone marrow, ≥ 20% basophils in the peripheral blood, platelets <100 x10^9/L not treatment related or platelets >1000 x10^9/L unresponsive to treatment, additional chromosomal abnormalities occurring on treatment, white cell count and spleen size increasing and uncontrolled on treatment (1). Response to treatment is also considered. Blast phase is defined as ≥ 20% blasts in the peripheral blood or bone marrow or extramedullary blast proliferation (1). Our patient had 23% blasts in the bone marrow and 26% in the peripheral blood therefore meeting the WHO criteria for blast phase disease.

Early information in this case was able to be obtained from the WDF scattergram from the automated haematology analysers. As the patient's WBC was markedly increased the results were flagged for validation and phoning by a scientist as per laboratory protocol.

Whilst awaiting blood film review, we were able to review the white cell differential scattergram for any information regarding a likely diagnosis. The presence of a large number of cells in the neutrophil and immature granulocyte region, the clear eosinophilia, the patient's markedly increased WBC and preserved indices somewhat suggested a potential CML.

However, the large population of cells sitting in the blast region is not typical of a scattergram seen in patients with CML-chronic phase. The scattergram shown on the left in Figure 4 is typical, but not exclusive to that seen in a patient with CML-chronic phase. Distinct populations can be seen as shown in colour with a large number of cells in the region of neutrophils (light blue) and immature granulocytes (dark blue) as well as an eosinophilia (red). Unlike our patient there are no cells found in the blast region as would normally be the case in a patient with CML-chronic phase. The heterogenous population of cells seen in the scattergram of our patient (shown on the right in Figure 4) and the presence of abnormal cells in the blast region may give an early indication of CML-blast phase. This was confirmed by the presence of a large numbers of blasts seen in the patient's blood film.

Upon review of the patient's previous CBC results, note was made of a mild persistent thrombocytosis and neutrophilia. An eosinophilia and raised red cell count were present intermittently. This raises the possibility that the patient's blast phase may have arisen from a previously undiagnosed CML-chronic phase. It is of note that in this case our patient previously demonstrated a borderline basophilia that was not overt. It was, however, higher than his previous counts and was at the upper range of normal. His eosinophilia was also intermittent.

This case highlights the importance of early detection and treatment of CML in chronic phase. With the use of TKIs treatment of CML-chronic phase has excellent response rates and overall survival rates. Patients who are treated in chronic phase have an overall survival similar to those of the general population (10). Some patients can achieve deep molecular remission and can discontinue treatment. The majority of patients, however, remain on TKIs indefinitely (10). Lack of efficacy and intolerance in some patients may lead to discontinuation of treatment (10). Progression to accelerated or blast phase occurs in approximately 5-10% of cases even with treatment (4,14). Patients with CML-blast phase are treated with TKIs with or without chemotherapy (15). Those with *de novo* accelerated phase disease should be treated with a second generation TKI. The patient presented in this case was treated with Dasatinib. Dasatinib is a second generation TKI which targets both *BCR-ABL1* and SRC- family kinases and has the ability to bind to active and inactive forms of *ABL* kinase (2,4). Dasatinib has been shown to be more effective than Imatinib against *BCR-ABL1* and is effective against most *BCR-ABL1* mutations (2,4). In patients that achieve a haematological response, subsequent allogenic stem cell transplant (SCT) should then be considered (15). Studies have shown patients with CML-BP who have undergone allogenic SCT following TKI therapy had a significantly higher overall survival than those treated with TKI alone (4). Relapse and resistance to TKIs is common in CML-blast phase and long-term overall survival rates remain low. The main goal for management and treatment of CML is to prevent the development of CML-blast phase from occurring in the first instance (16,17).

Very few patients present in blast phase disease and typically present with a more severe clinical picture than was seen in our case. Patients in accelerated or blast phase disease present with a clinical picture similar to an acute leukaemia with constitutional symptoms, such as fever and weight loss or symptoms related to anaemia or thrombocytopenia (1,6). Our patient presented to the emergency department with unrelated clinical issues and neither anaemia nor thrombocytopenia were present. He was, however, noted to have splenomegaly on examination and on reflection some night sweats and unintentional weight loss. The patient has had a good response to treatment which is unexpected in patients with accelerated or blast phase disease as they are more likely to be resistant to treatment and have a poorer outcome (4,11). The patient has shown an early molecular response with his *BCR-ABL* transcriptase levels reducing well over time however the patient's overall survival remains to be seen.

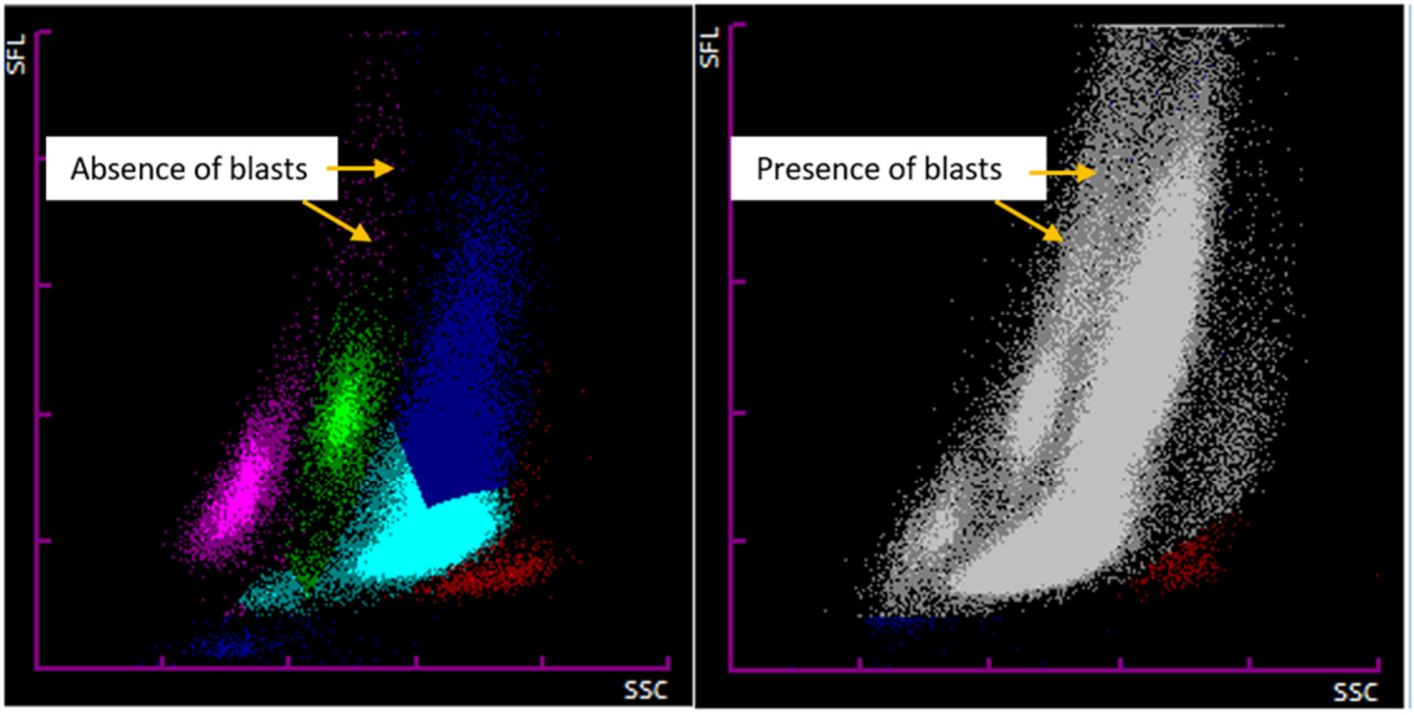

Figure 4. An example of a white cell differential scattergram seen in a patient with CML-CP on the left with an absence of blasts. An example of our patient's WDF scattergram on the right with increased numbers of blasts present and heterogeneous 'greyed out' population.

CONCLUSIONS

Nearly all patients diagnosed with CML present in chronic phase disease. Our patient was one of the rare cases that presented and was diagnosed in blast phase disease. Up to half of patients with CML are asymptomatic and diagnosis may be an incidental finding from routine blood work. This places importance on the role the laboratory scientist plays in the diagnosis of CML. As treatment in CML-chronic phase is extremely effective it is important to detect cases early in the course of the disease to ensure prompt treatment and a more favourable outcome. Even when presenting in situations where the picture can be explained by an acute setting there may be potential flags for an underlying myeloproliferative neoplasm, such as persistence of neutrophilia and immature granulocytes or accompanying abnormalities such as eosinophilia or basophilia. Recommendations for repeat CBC testing when patients are well with review of all previous CBC results would be appropriate in these instances to detect potential myeloproliferative neoplasms that have a chronic clinical picture.

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SCIENTIFIC LETTER

Zoom appears to cause malfunction of the TEG®5000 Thromboelestographic Hemostasis Analyser System when co-installed

Tara BM Feeley and Andrew B Meisner

ABSTRACT

The use of a single device to display thromboelastogram results and a Zoom telecommunications channel may result in an error in the processing of the blood sample. We used a single personal computer to display blood samples processed by the TEG® 5000 Thromboelastograph Hemostasis Analyser System, while using the same personal computer to host a Zoom meeting. A flat line was produced by the sample; however this did not correlate with the clinical scenario or with simultaneous laboratory coagulation results. We hypothesize that there is an incompatibility in running Zoom and the TEG® 5000 Thromboelastograph Hemostasis Analyser System together, and this may be due to the use by both applications of Microsoft's dynamic-link library.

Keywords: Point-of-care systems; COVID-19; Thromboelastography; Telecommunications; Quality Control; software.

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INTRODUCTION

We would like to share our experience of an apparent malfunction of the TEG®5000 Thromboelastograph Hemostasis Analyser System (TEG®5000) used in our operating department, and to alert our colleagues to the potential interaction between the Zoom videoconference platform software and TEG®5000.

Due to the requirements to minimise movement in and out of our department's COVID-19-dedicated operating theatre, communication between the clinical team inside the operating theatre and the support team outside the theatre was facilitated by an open 'Zoom' telecommunications channel. As in many clinical areas, the availability of information technology equipment is limited in our operating department. Thus, the Zoom application was installed on the same personal computer (PC) as is used for the TEG®5000 to display its results. Our software is 'Teg V4.2' as supplied by Haemoscope and installed locally on the PC.

Quality control (QC) of the TEG®5000 instrument involves daily electronic quality control (QC) and weekly liquid QC (which mimics a patient sample). On the morning of our software malfunction, an electronic QC was performed followed by a liquid QC. Both sets of results were as expected. A 'Zoom' session was not initiated prior to running the electronic and liquid QC.

Following this, a patient who was suspected of being COVID-19 positive was cared for in the dedicated theatre. Zoom was opened on the aforementioned PC and used for communication during this case, and a blood sample was processed with TEG®5000 for this patient. The TEG appeared to malfunction – showing a continuous flat line. Laboratory coagulation tests sent at the same time as the thromboelastograph sample did not correlate with the flat line shown on TEG®5000 analysis. The flat line result produced by the TEG®5000 was thus assumed by clinical staff to be a spurious result and the theory of the TEG®5000 malfunctioning due to the concurrent use of the PC for Zoom was proposed. As a result, our point of care engineers rebuilt the PC, reloaded Windows TEG®5000 software and removed the Zoom software. An additional PC was installed in our department for use with Zoom. Since this time, the same TEG®5000 machine has run without issue.

We have contacted the TEG®5000 vendor (Haemonetics) and explained this occurrence. To date, we have had no response from them. The QC sample was normal – most likely because although Zoom had been installed it had not yet been opened on this PC. We hypothesize that this incompatibility may be due to the use by both TEG®5000 and Zoom of Microsoft's dynamic-link library. Dynamic-link library is Microsoft's module that contains functions and data that can be used by other applications; it is a means of modularizing applications that also reduces memory overhead.

Resources are limited in healthcare settings internationally. We are certain that our experience may well be repeated elsewhere, with concurrent use of one PC for two such commonly used applications - TEG®5000 and Zoom. We wish to publicize our experience in order to prevent misinterpretation of a spurious thromboelastograph result in other critically ill patients. We would caution our colleagues against installing Zoom on the same PC as TEG®5000. Additionally, it would be prudent for medical laboratory personnel to always open any newly installed software or applications prior to running quality control samples in order to prevent a repeat of our experience.

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The Pacific Pathology Training Centre 1996—2021

Philip Wakem and Ron Mackenzie

The Trustees and staff of the Pacific Pathology Training Centre (PPTC) take pleasure in extending congratulations to the New Zealand Institute of Medical Laboratory Science (NZIMLS) on the occasion of its 75th anniversary. The PPTC acknowledges with thanks the long and special partnership it has enjoyed with the Institute since the PPTC was first established on the Wellington Hospital Campus some 40 years ago.

The PPTC is recognised internationally as a centre of excellence and a Collaborating Centre of the World Health Organization) (WHO). In this role, the PPTC delivers relevant and efficient support for regional health laboratory training and quality assurance, and is proud to be a small but significant part of the New Zealand Overseas Development Aid Programme.

With ongoing assistance from the NZIMLS, the New Zealand Government's Overseas Development Programme, New Zealand Red Cross, and the Norman Kirk Memorial Trust, the PPTC fulfils a function which is not available through any other regional organisation or institution. Its programmes are both effective and unique and are an important element in strengthening the Pacific and South East Asian clinical, public health and blood transfusion services. The narrative which follows gives an account of PPTC activities during the years 1996 - 2021.

PPTC EQA Programme

The PPTC External Quality Assessment (EQA) programme has provided its quality service to the laboratories of the Asian – Pacific region for over 30 years. Since 1990, the PPTC has been recognised by WHO as a Collaborating Centre in its role as an EQA provider and driver of quality improvement initiatives. By 2002 the PPTC's EQA programme had become a major activity in the Pacific region with 21 laboratories participating, 19 of which were National Laboratories whose participation at this time was supported by WHO. Laboratories at Lae (PNG) and Qui Nhon Provincial Hospital (Vietnam) were sponsored by private arrangement.

In 2002, the PPTC became involved in reviewing smears for tuberculosis (TB) sent from laboratories in Samoa, Kiribati, and Tonga. This activity was the outcome of a review carried out by WHO and SPC (Pacific Community) of the TB laboratory services in these countries and had the aim of further developing TB diagnostic and treatment services in line with WHO's Directly Observed Treatment Short Course (DOTS) Programme. In 2002, the PPTC was recognised by WHO and SPC as a reference laboratory for TB EQA and given official responsibility for monitoring the laboratory TB work in Samoa, Tonga, Kiribati, Niue, Tuvalu and the Cooks.

As the years rolled by the PPTC's EQA programme not only expanded in the number of registered Pacific Island laboratory participants, but also in the diversity of quality assurance coverage for the main medical laboratory disciplines. Haematology, biochemistry, microbiology, blood transfusion, and infectious disease serology were the major disciplines covered within the EQA programme up until 2018 after which, anatomic pathology (2019) and molecular COVID 19 (2020) extended the programme even further.

In 2005 the PPTC EQA programme expanded to 27 laboratories. In 2008, 35 laboratories registered and participated. This further increased in 2014 to 40 registered laboratories and continued to climb to 65 laboratories in 2015 Pacific wide. By 2019 the total number of laboratories on the PPTC's EQA programme rose to 101 and plateaued back to 86 in 2020/2021 as a result of reduced funding for specific Cambodian laboratories.

In 2003 the Centre was approached by the South Pacific office of WHO in Suva to prepare and deliver a trial course through its newly set-up distance learning website, Pacific Open Learning Health Net (POLHN). A course was developed in the "Laboratory Diagnosis of Bacterial Diarrhea" and delivered in mid-2004 to laboratory personnel from various Pacific Islands. Following the success of this initial course, the Centre was again contracted by WHO to deliver a second course in June 2005. This second course was an introductory course in clinical biochemistry and there were 33 laboratory staff registered for it.

For a number of years the PPTC staff and Board were concerned about the lack of basic training in medical laboratory science available to the majority of staff working in health laboratories of Pacific Island countries. With the advent of POLHN, a system became available to commence addressing this shortcoming. As a result, discussions were held with WHO that lead to the PPTC developing and delivering a "Diploma" course covering the basic medical laboratory sciences; microbiology, biochemistry, haematology, transfusion science, and immunology. Modules were developed in these five sciences and this POLHN programme has continued since its introduction in 2006.

In 2010, as a result of ongoing evaluation, it was proposed by the PPTC and accepted by WHO, that the Diploma programme be revised in terms of both its theoretical and practical component, with the introduction of log books as a means towards the extension of practical skill and a measure of practical competence. At the beginning of 2011 a series of enhancements were introduced. The theoretical content of each of the modules underwent a comprehensive review, with curricula expansion and updates applied to each of these modules in order to reflect recent medical advances.

The immunology module that featured in the initial Diploma (pre-2011) was discontinued and instead its contents incorporated into both the microbiology and transfusion science modules. This made room for the development of a laboratory technology module which became its replacement and was considered by the PPTC to be an essential addition to the programme in order to address the need for a base understanding of the fundamental principles of the biological, chemical, and physical sciences on which the medical laboratory sciences are built.

A second major change to the programme was the addition of an accompanying log book to the modules of haematology, biochemistry, microbiology and transfusion science, each of which required signoff by the Charge Technician or Head of Department once completed. As a result of this, instead of a six-week completion time for each module studied as had been the case in the past, each module now would take three to four months to complete. Each log book not only details the practical tasks to be carried out, but also contains a great deal of valuable information associated with each routine procedure. Delivery of this revised teaching programme commenced in 2011 as a two-year study programme, the completion of which was scheduled for the end of 2012. The number of students who registered for the various five POLHN modules in this 2011 -2012 cycle totalled 96, with 30 students progressing towards the completion of the Diploma, while the remaining 66 registered for individual modules only, as a professional development exercise. Also in 2011, the Fiji School of Medicine (FSM) gave recognition to the PPTC Diploma granting credits for selected units in its BMLSc programme.

In early 2012, the Diploma was renamed the Diploma in Medical Laboratory Science (PPTC) from the existing Diploma in Medical Laboratory Technology (PPTC). A second cycle of the revised Diploma was launched in March, 2013, and completed at the conclusion of 2014. In 2013, the PPTC proposed a further change to the scope of the Diploma with the addition of a laboratory quality management systems (LQMS) module. This increased the number of modules offered in the DipMLS (PPTC) to six, with the new LQMS module offered as the final module in the Diploma programme in year two. The Diploma currently consists of 6 modules:

1. Laboratory Technology
2. Haematology
3. Biochemistry
4. Microbiology
5. Transfusion Science
6. Laboratory Quality Management

The final review of the Diploma Programme took place in 2013 with the introduction of two examinations, Part 1 and Part 2. Part 1 examines the first three disciplines (laboratory technology, haematology, and biochemistry) and Part 2 examines the second three disciplines (microbiology, blood transfusion, and laboratory quality management).

The course, is designed to nurture and guide students through the program beginning at the very basic level of theoretical content, graduating through an intermediate level and finally onto advanced achievement, enabling them to reach levels of excellence in competence and performance as laboratory professionals. From 2013, and the years that have followed, the PPTC's Diploma, which is now funded in its entirety by the New Zealand Government at no cost to the student, continues to be a valued educational experience for Pacific students throughout the region. To date, 115 students have graduated with the PPTC Diploma of Medical Laboratory Science.

PPTC Centre Based Courses

Centre based courses that were provided at the PPTC in Wellington over the last 25 years for visiting students from both the Pacific and the South-East Asian region varied in terms of discipline. Blood bank was offered each year while biochemistry, haematology, microbiology, quality management, and infectious diseases alternated. Between 1996 and 2011 an average of two to three courses were offered each year and in the years to follow this was increased to an average of five to six courses per year until the beginning of 2020, when the impact of the COVID pandemic became a reality and prevented overseas students from studying in New Zealand. The number of students who attended classes in NZ between 1996 and 2019 totalled 416.

In Country Training

Apart from New Zealand based courses, the PPTC's provision of training courses held in specific countries and in specific disciplines has been an integral component of its Pacific commitment since its inception in 1980. These in-country training programmes have been funded by various bilateral and multilateral agencies including the New Zealand Ministry of Foreign Affairs and Trade, the NZ Red Cross, WHO, SPC, UNDP (United Nations Development Programme), NZ Vietnam Health Trust, NZIMLS, and PIHOA (Pacific Island Health Officers Association).

Other funders and support groups include the Pacific Ministries of Health, the Norman Kirk Memorial Trust, the New Zealand Blood Service, the Royal College of Pathologists of Australasia, the University of Otago, NZIMLS, and Southern Community Laboratories. PPTC consultants have carried out numerous training assignments in countries such as Papua New Guinea, Federated States of Micronesia, Marshall Islands, Fiji, Niue, Tonga, Vanuatu, Kiribati, Timor-Leste, Samoa, Cook Islands, Solomons, Cambodia, Philippines, Vietnam, India, Nepal, Bhutan, Mongolia, American Samoa, Tokelau, Tuvalu, Nauru, and Palau. Over the last 25 years, teaching and training activity in - country has focused very much on TB diagnostic procedures and surveillance, external quality assurance, laboratory quality management, discipline related strengthening, blood transfusion services, infectious disease diagnosis and monitoring, international accreditation, Pacific laboratory capability and capacity building, and portable laboratory construction. Dedicated PPTC staff, voluntary tutors, and external consultants who have contributed tirelessly to achieve the PPTC's mission since its inception in 1980 are to be credited with the continued success the PPTC carries as a development partner and the recognition bestowed upon it as a "Centre of Excellence" as a provider of education and training in the Medical Laboratory Sciences.

A time line of changes to the PPTC over the last 25 years

1997: The PPTC building is relocated on the Wellington Hospital campus.

2000: Mike Lynch retired from the PPTC after 15 years as tutor coordinator. John Elliot was appointed as his successor.

2005: The PPTC celebrated 25 years of operation in the Pacific.

2008: Phil Wakem joined the PPTC as Programme Manager for all teaching and training programmes.

2010: PPTC celebrated 30 years of operation in the Pacific.

2011: Alterations to expand the PPTC laboratory were completed. Dr Ron Mackenzie retired as Board Chairman after 30 years of service to this position. Mike Lynch is elected Board Chairman of the PPTC.

2012: After completing 11+ years as Director of the PPTC, John Elliot retired on the 3rd February**.** Phil Wakem was appointed Manager of the PPTC. Ruth Reeve (Medical Laboratory Scientist) was appointed to the PPTC Board. Russell Cole and Navin Karan were employed as permanent PPTC consultant staff.

2013: Christine Story retired from the PPTC as Administrative Manager on 17th May after 30 years of service. The PPTC Board of Management welcomed Filipo Faiga as its newly appointed co-opted Board member.

2014: Marilyn Eales retired from PPTC as a Board Member. The PPTC Board of Management welcomed John Elliot as a PPTC Board member. Phil Wakem was re-designated as Chief Executive Officer of the PPTC.

2015: Clare Murphy retired from her position as PPTC EQA Consultant for biochemistry.

2016: The PPTC was awarded a five year grant funding agreement with the New Zealand Ministry of Foreign Affairs and Trade. Ruth Reeve retired from the PPTC as Board member. Filipo Faiga was appointed permanent staff member and biochemistry Technical Specialist to the PPTC.

2017: The PPTC Board of Governance, as it was now known, welcomed Angela Brounts and Marion Clarke as Board members.

2018: Launch of Dr Ron Mackenzie's book "PPTC - The first 30 years, 1980 – 2010"**,** Mike Lynch retires from the position of PPTC Board Chairman. John Elliot elected as PPTC Board Chairman. Vichet Khieng was appointed permanent staff member as medical laboratory and IT specialist to the PPTC and to the Pacific.

2019: The PPTC changed from an Incorporated Society to a Charitable Trust and changed its name to the Pacific Pathology Training Centre from the PPTC.

2020: Angela Brounts and Mike Lynch retired as Trustees of the PPTC Board. Vichet Khieng resigned from the PPTC. The PPTC Trustees welcomed Dr Vladimir Osipov and Dr Dianne Sika-Paotonu as Trustees.

International meetings

Between the years of 1996 and 2019, staff of the PPTC travelled extensively on the International circuit and attended numerous conferences involving such organisations as Red Cross, WHO (Geneva), PPHSN (The **Pacific Public Health Surveillance Network) (**Noumea) , PIHOA, SPC, the Pasteur Institute (Noumea), WHO CDC (The **Center** for Disease Control and Prevention) (France), WHO (Manila), WHO (Fiji), WHO (Vietnam).

At the request of Red Cross in 1999, both Dr Ron Mackenzie and Mike Lynch presented papers at the Pacific regional blood banking seminar in Port Moresby Papua New Guinea, and in the same year, Ron spoke at the International Society of Blood Transfusion conference in Lucknow, India, and Mike Lynch spoke at a WHO meeting of WHO Quality Assurance providers at WHO Headquarters in Geneva.

In 2000 the PPTC was invited to participate in the inaugural meeting of the PPHSN held at the SPC headquarters in Noumea and in 2006, John Elliot and Christine Story attended an SPC / WHO/ Pasteur Institute five day workshop in Noumea to discuss and clarify the technical aspects of laboratory testing currently available for the diseases targeted by the PPHSN. (typhoid fever, cholera, influenza, dengue, leptospirosis and measles)

In November 2007, the PPTC was asked to join the NZ Ministry of Foreign Affairs and Trade's 2007 Pacific Island Mission. The delegation, headed by the Minister of Foreign Affairs, Winston Peters, was made up of Parliamentary members, Ministry officials, academics, representatives from NGO's, and journalists. Associate Professor Rob Siebers represented the PPTC and the event was considered very valuable in terms of public relations and networking.

In 2008 John Elliot was invited to attend a WHO-CDC conference in Lyon, France, the focus of which was health quality systems. This gave an excellent opportunity for John to speak on the PPTC's REQA programme in the Pacific region. In the same year, SPC and WHO held a joint meeting in Pago Pago, American Samoa to review the laboratory diagnosis of HIV and other STI's (sexually transmitted infections). John Elliot attended this meeting as a technical advisor and was appointed to the working group which would complete the review and make recommendations on improving the reliability and efficiency of STI and HIV testing especially. Following this meeting, John Elliot, once again as technical advisor / rapporteur, was requested by WHO to join an international team in Hanoi which was developing guidelines for HIV laboratory testing in the Western Pacific region. Towards the end of 2008 John Elliot attended a WHO meeting as a technical advisor to review a strategy document entitled "Asia Pacific Strategy for Strengthening Health Laboratory Services 2011-2015".

In 2010 WHO convened a meeting in Suva to finalise a document giving guidelines for the development of a National Laboratory Policy and Plan. This meeting was attended by representatives from most Pacific Island countries as well as PPTC consultants, including Phil Wakem and John Elliot. Representatives also from the NZ Ministry of Foreign Affairs and Trade, SPC, PIHOA, NRL (National Serology Reference Laboratory), and CDC Atlanta were present. A second document written by the PPTC, proposing a set of standards based on the international ISO15189 standard but applicable to the region was also introduced and discussed. In the same year, John Elliot attended a meeting convened by the Fiji School of Medicine to review the education and professional development of the medical laboratory workforce in the Pacific. Along with the PPTC and the Fiji School of Medicine, SPC and PIHOA were also in attendance. Following this meeting, a Memorandum of Understanding was signed between the Fiji School of Medicine and the PPTC, recognising the PPTC Diploma, and granting credits for selected units in their established BMLSc programme.

In 2011 John Elliot and Phil Wakem attended a WHO meeting held in Fiji from 14th to 17th September. Attendees included representatives from most Pacific Island countries and in addition observers from NZAID, SPC, PIHOA, NRL and CDC, were also present. The aim of this forum was to assist Pacific countries draw up national plans and policies for laboratories. In 2013 WHO invited Russell Cole, the PPTC's senior consulting microbiologist, to participate in an international technical workshop, the focus of which centered on antimicrobial resistance surveillance in the Western Pacific Region.

In 2014 Phil Wakem visited Manila to attend an international forum of the WHO Collaborating Centres for which he was asked to present an update on the PPTC's work and its contribution to Pacific health within the region. Over 200 participants from 124 WHO Collaborating Centres located in the Western Pacific Region attended this forum.

In 2015 Navin Karan, as Manager of the PPTC's EQA Programme, attended a training workshop provided by WHO in Nadi, Fiji which addressed shipping requirements for all potentially infectious substances, focusing particularly on highly infectious materials.

The 2nd WHO Collaborating Forum meeting, held in Manila, Philippines in November 2016, built on the gains of the first forum by further developing inter-professional approaches to collaboration and partnerships to help countries meet the sustainable development goals (SDGs). 260 delegates were invited to attend and Phil Wakem and Navin Karan who represented the PPTC were asked to provide a presentation on the PPTC and its distance learning programme through POLHN, which was well received.

A WHO initiated meeting was conducted in Manila, Philippines in June 2018, the objective of which was to discuss current status and issues involving viral hepatitis laboratory services in the Western Pacific Region with the development of recommendations for improving quality of laboratory services in Pacific countries. This meeting was also responsible for "terms of reference" development of the regional viral hepatitis laboratory network, including roles and responsibilities of Regional/National Reference laboratories. Blood safety was also addressed. The PPTC was invited as an advisor to present on its EQA Program and the laboratory support it provides to resource limited countries. Navin Karan, the PPTC's programme manager and microbiology consultant represented the PPTC at this meeting and contributed to the presentation of specific aspects of EQA in the Pacific.

As part of the CDC support in strengthening influenza testing and surveillance in the Pacific, SPC invited the PPTC to attend a one-week training workshop on influenza like illness and hospital-based severe acute respiratory illness surveillance in Suva, Fiji in August 2018. Navin Karan represented the PPTC at this meeting and presented to those in attendance, the use of the Gene Expert in Pacific Island laboratories as well as a comprehensive overview of laboratory quality management systems. Also in 2018 Phil Wakem was invited to participate in the inaugural meeting for the Pacific Islands Society of Pathologists held in Suva, Fiji during September. The meeting was jointly sponsored by SPC and the Fiji National University (FNU, previously Fiji School of Medicine) and consisted of presentations from participating Pacific Islands countries and external agencies as well as group work.

Towards the end 2018 Phil Wakem was invited to attend the 3rd WHO Collaborating Forum, but on this occasion, Ho Chi Minh City, Vietnam was the nominated host country. The focus of this meeting was to (1) share good practices and reflect on progress since the second forum in 2016, (2) strengthen and promote innovative collaboration and networking mechanisms and (3) identify opportunities to maximize contribution of WHO Collaborating Centres towards WHO support at the country level.

In 2019 Phil Wakem and Navin Karan attended the 6th Association of USAPI (United States affiliated Pacific Islands) Laboratories, and PIHOA LabNet meeting in Guam. The theme "In pursuit of excellence in quality USAPI laboratory services" provided the basis for both Navin and Phil to present "Updates on medical lab workforce development, EQA and LQMS in the Pacific". In the same year Russell Cole and Navin Karan were both invited to the PPHSN's regional meeting held in Nadi, Fiji in June, the theme of which was "Linking up the initiatives and scaling up the actions". The conference gave the delegates present the chance to share views, successes and challenges on laboratory matters likely to hinder disease surveillance.

During the conference, Russell as chairperson of the LabNet Technical Working Body, was given the opportunity to update on progress made through LabNet, as well as present on the PPTC's "Laboratory Service/Accreditation Development programme".

2020 to the present

COVID 19 continues to threaten the Pacific

The global pandemic has had a serious impact on PPTC operations, causing a sizable disruption to our overseas in-country training programmes, and to the Wellington centre based courses, due to international border closures. It was, however, fortunate that both the PPTC's EQA Programme and the Diploma programme continued to be delivered relatively uninterrupted.

With the virus spreading globally, all efforts are being made to ensure that countries are prepared and ready to respond, in situations where COVID infection has been detected. In the Pacific, countries are increasing their efforts to ensure that their health services have reached an acceptable level of preparedness to rapidly detect and respond to the threats of COVID-19. The PPTC is continuously assisting in Pacific preparedness against COVID-19 and is currently working with WHO, New Zealand Ministry of Foreign Affairs and Trade, Pacific Ministries of Health, SPC, the Australian Department of Foreign Affairs and Trade, and the Doherty Institute in Melbourne to establish COVID-19 diagnostic and treatment facilities in Pacific countries, the majority of which would face the devastating effects of COVID-19 in the event of community spread.

In response to a request by the Niuean Government, WHO commissioned the PPTC to design and oversee the construction of a mobile/container laboratory that would deliver a comprehensive medical laboratory testing service, a service that would increase clinical diagnostic capacity, capability and sustainability through the provision of a broad spectrum of diagnostic testing addressing both non-communicable and communicable diseases, including COVID-19, to Niue's Foou Hospital. The PPTC through its specialist expertise, extensive experience and organisational skills accepted the commissioning by WHO and provided a container laboratory system that had a sufficiently large footprint to provide services, including phlebotomy, biochemistry, haematology, microbiology, serology, molecular analysis, blood transfusion and biosafety, across two facilities. Added to this responsibility was the selection, purchase, installation, validation, and verification of appropriate items of diagnostic instrumentation and associated laboratory consumables that would construct the required testing scope the laboratory would provide.

The New Zealand Ministry of Foreign Affairs and Trade granted the PPTC ongoing opportunities to develop new skills in terms of laboratory refurbishment and container laboratory construction. Not only did the Ministry provide the funding to the PPTC to upgrade the Samoa hospital laboratory (2019) in Apia in response to the measles outbreak there, but also for the construction and complete refurbishment of a portable laboratory container system for the Tokelau's (2020) for which the PPTC again was commissioned. Following this, the PPTC was further commissioned by the Ministry to assist in the construction of a container laboratory system for Kiribati (2020) and the setup of an existing laboratory in the Cook Islands (2021), both for COVID-19 testing.

Exterior of the container laboratory commissioned for Niue

In 2021, preparedness against COVID-19 infection and community transmission was addressed by the Ministry of Health & Medical Services of Fiji (MOHMS) in their request for the construction of a mobile/container laboratory to be established in Nadi, capable of RT-PCR testing and a range of other necessary tests near this port of entry. It was envisaged that this newly constructed laboratory would undertake up to 200 COVID-19 tests a day by RT-PCR. In response to this request, the PPTC was commissioned by WHO to oversee the design and construction of a mobile/container laboratory, with the implementation of appropriate validated/verified equipment to deliver the required testing menus, relevant training of staff, and the provision of reagents and kits on a regular basis to deliver the medical laboratory service for Nadi.

The recent measles outbreak in Samoa and the current COVID-19 pandemic have highlighted many deficiencies in the health sector, particularly laboratory diagnostic services within the Pacific region. The PPTC's expertise in general laboratory strengthening and medical laboratory diagnostic services is more relevant than ever to the regional health status, especially to help protect the region from infectious disease outbreaks and to enable early detection of chronic diseases.

CONCLUSION

In essence, the training and quality improvement programmes of the PPTC and its work on developing regional laboratory standards has proved highly relevant in strengthening the capacity of Pacific Island countries in the provision of clinical and public health laboratory services and align well with the WHO Asia Pacific strategy.

The PPTC has now entered its 4th decade and re-affirmed the basic principle on which it was founded *"The provision of appropriate affordable sustainable technical training and assistance for the medical laboratory and blood transfusion services of the Pacific Islands and South-East Asia".* With continuing and valued support of the NZIMLS, the PPTC looks forward to the new and different challenges the 4th decade will bring.

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75TH ANNIVERSARY ARTICLE

Evolution of haematology 1996 – 2021

Steve Johnson

When I was asked to contribute this article describing how haematology has changed over the last 25 years (1996 – 2021), I realised that the timeframe fitted in nicely with the second part of my career as a haematology laboratory scientist. Prior to 1996, laboratories in New Zealand were either community laboratories who performed laboratory testing and received payment for the service on a fee per test basis set out in a schedule of allowable tests; or hospital laboratories that were owned and directly funded by the government. The community laboratories that had been traditionally owned by pathologists were being acquired by national or international companies. In the early 1990s, SGS (a Swiss global certification company) acquired Medical Diagnostics, the laboratory I was employed by. Medical Diagnostics had new management who viewed change as an opportunity and used innovation as a tool to chart their course through the challenging times to come. The following description of the last 25 years is as seen through my eyes, but I am sure that there will be many events, circumstances and technologies that will be familiar to most readers.

In 1996 (as with now) there was a desire to make the health dollar go further and as a result the Crown Health Enterprises (CHEs), which were the predecessors to today's DHBs, started to consider contracting out their pathology services. Palmerston North Hospital chose this option, and our laboratory was successful in winning the contract. We became Medlab Central and set up to provide the pathology services for both hospital and community patients from the one laboratory site at the hospital, the first laboratory in New Zealand to do this. This set the scene for the changes that we were to encounter over the next 25 years. These changes fall into three categories: changes in remuneration for services and testing rationalisation; incorporation of information technology into most aspects of the laboratory, and the development of new scientific techniques.

Changes in remuneration for services and testing rationalisation

With the drive to maximize the value of the health dollar, CHEs and subsequently DHBs moved from fee for service-based funding to bulk funding (capped budgets). Funding organisations provided educational resources to encourage clinicians to rationalise laboratory test requests, thus there was a desire for demand management in laboratory medicine, the desired outcome of which was not only to reduce demand but also to contribute to better medical practice and optimal use of the laboratory as a resource.

There wasn't a marked change in practice from the users of the laboratory service in terms of tests being requested and so laboratories needed to find ways to discourage unnecessary test requests whilst maintaining and indeed improving the service to the clinicians. As the sole laboratory in Palmerston North and having a good rapport with our clients, we could effect changes at various points of our interface with them in order to streamline the use of the laboratory service.

The first opportunity to do this was when we redesigned the hospital request form. Up until this point, tests were often grouped into panels of tests which would be ticked, and all the component tests would be performed regardless of whether they were justified or not. The body of the new form was a series of blank columns under laboratory discipline headers – Haematology, Biochemistry, Microbiology, etc. We included tick boxes for whether the patient was on heparin or warfarin so that unnecessary follow-up testing wouldn't be performed in the case of an abnormal result that was due to anticoagulation. While this change had the desired result in the long run, there were attempts to revert to previous practice. An example was some enterprising emergency department clinicians getting rubber stamps of panel tests made up which they would duly stamp on forms and send to the laboratory with the specimens.

There was reluctance to provide clinical information (surprise, surprise) and locations such as ICU wouldn't provide anticoagulation details but wanted complete results provided in a very short timeframe. This reluctance continued even after discussions with them about how providing this information could mean we would possibly have to perform fewer tests, resulting in a shorter turnaround time. We were able to resolve this in later years as we moved to electronic ordering which I will elaborate on in the information technology section later in this article.

Another opportunity to change requesting patterns was in 2010 when we noticed that ESR was frequently requested in conjunction with CRP which we felt was a redundant use of a test. We were also concerned that ESR had no uncertainty of measurement possible for moderately or markedly elevated human samples and no QC possible except on stabilised specimens which did not behave in the same way as human specimens. After discussion between senior laboratory staff and haematologists, we sent a memo out to all clinicians that ESR would only be performed when requested in five clinical scenarios (later expanded to eight). Despite initial pushback from many clinicians, this became accepted and our daily numbers of 200 ESRs pre-2010 reduced to three – four after acceptance of the limited testing protocol.

Very soon after we introduced triaging of requests for thrombophilia screening which up until then had been performed on demand. This was at a time when "*economy class syndrome*" had been topical and so request numbers were increasing. Complete thrombophilia screens were very expensive requiring coagulation studies, chromogenic tests, and molecular studies. All requests for thrombophilia screens would be forwarded to the haematologists (once clinical information had been provided) and they would report whether the tests would be performed or if not, why not. Our numbers of five – six per week dropped to less than one per fortnight. This required a lot of staff time putting samples aside, maintaining paperwork, and contacting requestors for clinical information, etc. however, this was subsequently streamlined very efficiently with the advent of middleware and subsequently electronic ordering, which segues nicely into the information technology changes we were to encounter.

Information technology

In the early 2000s CBC and coagulation analysers were quite well automated, enabling large volumes of tests to be performed with reasonable staffing levels. Racks of 10 specimens were able to be presented to the analysers and through the LIS interface, the analysers would know which tests were required and would subsequently perform them. However, there were still requirements for staff intervention with preanalytical checks such as for fill volume and haemolysis in the case of coagulation specimens; and checks of request forms for non-routine tests that may have been requested for EDTA specimens (Kleihauer, haemoglobinopathy, malaria, etc.). Enhancements to the LIS capabilities allowed us to forego checking request forms which was a huge efficiency gain. Improvements to coagulation analysers obviated the need for preanalytical checks by staff. The bottleneck then became the review of results once they were available from the analyser.

These all had to be checked for abnormal flags and while crude delta checks could be performed by the LIS, there remained many films that required review (45% – 50% in those days).

From 1996 - 2004, we had two standalone CBC analysers, an Advia 120 which accepted racks of 10 specimens (Figure 1), and a Technicon H*1 for back-up which had a bandolier specimen feed (Figure 2).

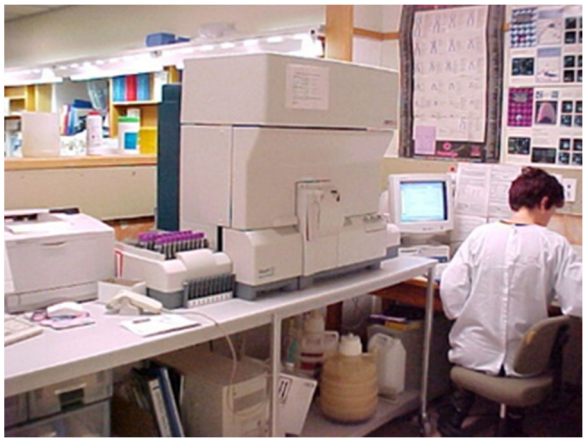

Figure 1. Advia 120 analyser.

Figure 2. Technicon H*1 (back-up).

Decisions to perform film review were made by manually checking CBC results against previous results, request forms, analyser scatters, and sorting film lists into urgent and routine (Figure 3); a process which was demanding on staff resources. Blood films were prepared manually and stained with a Hematek stainer (Figure 4).

Figure 3. Manual film review.

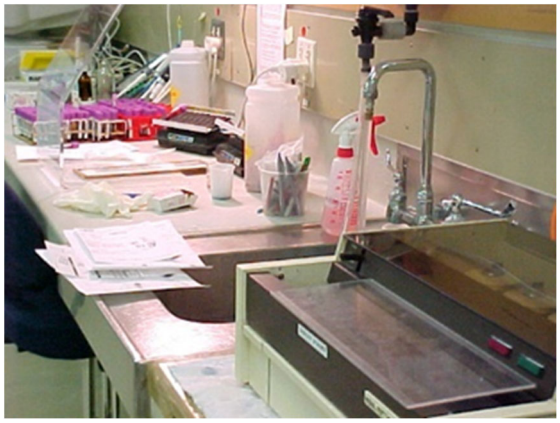

Figure 4. Manual stain bench.

In mid-2004 we upgraded to a Sysmex HST system which consisted of two CBC analysers with a slidemaker/stainer on an automated conveyor system (Figure 5) and this was subsequently upgraded in 2016 to our current Sysmex XN90121 system comprising three CBC analysers and a slidemaker/stainer also on an automated conveyor system (Figure 6).

Figure 5. Sysmex HST system.

Figure 6. Sysmex XN90121.

While this was a great improvement, the game changer was the implementation of middleware. With middleware we were able to devise a set of rules which would analyse the results and quickly report any normal or marginally abnormal CBCs. All flags were identified and where required, further testing such as reticulocytes or optical platelet counts could be implemented real-time.

We were able to perform much more detailed delta checks so that gradual deterioration in results would necessitate film review when a defined delta from the last film review had been reached. We could also perform delta checks on instrument flags which were now able to be seen as a numerical value where previously they were only seen as present or absent. Requestor locations were built into the rules so that if chemotherapy wards were the location, then a new neutropenia or thrombocytopenia wouldn't necessarily trigger the same requirement for notification or film review as a patient from other locations. Patient samples generating results that fell within our critical criteria would generate an alarm which prompted actions for notification and generated an audit trail.

Utilising middleware, specimens requiring film review were identified and the films prepared and stained by the analyser without human intervention; our film review rate also dropped from almost 50% to just over 20%. When staff were reviewing the films, they were able to see the analyser results and cytograms (in colour) on the middleware screen, where previously they only had the basic CBC indices to work with. Also, on the middleware screen were prompts for review identifying what rules had generated the film and what flags were present.

Thus, middleware had a huge influence streamlining the CBC component of our work and it was certainly no different for coagulation. Some analysers of the day had a basic capacity to perform reflex testing but with the advent of middleware we were able to get much more creative with rules and perform complex pathways for reflex testing where required. The investigation of abnormal coagulation results became automatic thus eliminating the possibility of human error, every result would get the follow-up it required and would be referred for haematologist review when necessary. Triaging of thrombophilia requests became much less cumbersome utilizing middleware. When clinical information was not provided on an initial request form, middleware would not allow the request to proceed to triage. An automated comment asked clinicians to provide clinical details which, if received, would enable triage. This was then a seamless referral to the haematologists who would decide on, and add, the required analyses and send an interim report to the clinician accordingly.

Middleware also allowed us to review QC and compare analysers from our main lab and our satellite labs. Westgard rules were available, alerts would flash up when an assay was out of range and the operator would record the actions taken against each offending result. We were able to view all results and middleware screens in the satellite laboratories which meant that referrals from these sites to the haematologist in the main lab were seamless, visible, and able to be tracked, thus minimizing the chances of anything going astray.

Sysmex ÉCLAIR (a clinical data repository) was developed in the early 2000s and implemented into our hospital not long after. It has grown to become a repository for laboratory results, pharmacy records, imaging records, and discharge summaries. The possibility to utilize an ÉCLAIR electronic orders module arrived and we decided to implement this into ICU as a pilot site. The aim was to streamline request ordering for a requestor who operated in a pressure environment and to gain efficiencies in the laboratory by removing the possibility of transcription errors and accelerating the specimens' passage through the front end of the laboratory. This greatly improved turnaround times but the game changer was being able to manipulate the requestor to provide the information we require. ICU was able to easily provide anticoagulation details so that extra testing was minimised, and turnaround times improved markedly.

When electronic ordering was introduced to community GP practices, we were able to further influence requesting behaviour. Where we wished to limit testing to specific clinical scenarios (such as ESRs as previously described), a drop box was included on the electronic request form for those tests so that the test was unavailable unless an appropriate indication was ticked. This reduced unnecessary testing and minimized staff involvement for triaging such as that described previously for thrombophilia screening.

New scientific techniques

In 1997 the WHO proposed a reclassification of haematological malignancies so that instead of using morphology as a basis for classification they would define disease entities that could be recognized by pathologists and that had clinical relevance. There was a shift from cytochemistry towards cytogenetics and newer technologies such as immunophenotyping. As subsequent revisions of the WHO classification appeared there was a focus on even more recent technologies such as molecular techniques (PCR, etc). As a result of this evolution we ceased performing labour intensive stains, such as Sudan Black, esterase, and acid phosphatase, and sent specimens to referral labs for the currently appropriate testing required.

Evolution in haematology analysers allowed new parameters to be measured and reported. One such parameter was the haematopoietic progenitor cell (HPC) which was a surrogate marker for CD34 positive stem cells. As a leukemia treatment centre we performed regular autologous peripheral blood stem cell (PBSC) transplants; however, we had no immunophenotyping to provide CD34 counts on site, instead relying on reference laboratories to provide this. The delay in turnaround times due to off-site testing made prediction of optimum stem cell harvest times and harvesting difficult compared to sites with on-site testing. The availability of HPC counts meant that we could turn around this testing in three minutes (compared to one – two hours with immunophenotyping) and have the same or better success than those using immunophenotyping.

Other novel parameters allowed us to detect disease states at an earlier stage. Examples are Neut-X (WDF-X) to flag neutrophil hypogranularity as seen in myelodysplasia and immature platelet fraction (IPF) to recommend JAK2 testing in cases of undiagnosed polycythaemia vera. These new techniques and parameters enabled us to not only gain efficiencies as far as staff resources were concerned but to be able to provide a better service to the clinician and patient by detecting disease states earlier, which could lead to improved outcomes for the patient.

In 2010 we saw the introduction of Dabigatran which was the first of the direct oral anticoagulants (DOAC). Subsequently Rivaroxaban arrived on the scene. Whereas warfarin had been traditionally used for anticoagulation, it was susceptible to changes in medication, diet and had large interpatient variability. For these reasons regular monitoring of coagulation status was required, and this could be weekly in many patients. The effects of DOACs were more predictable and so patients on these medications didn't require monitoring and test numbers began to fall. One drawback was that when patients acutely presented to Emergency Departments, their medication history was often not immediately available and so tests were needed to detect the presence of DOACs especially in a pre-surgical setting. For this purpose, thrombin clotting time (TCT) and protamine sulphate TCT were used to detect Dabigatran and an assay was developed in-house for Rivaroxaban.

As has always been the case with medical laboratory science, the only thing that is constant is change and our profession has developed to succeed in these situations. The varying political climate bringing restructuring of the health system and its associated fiscal changes of the past 25 years is still occurring today with the plan for a major reform of the health sector replacing the 20 DHBs with a single health authority. Change may be viewed either as a threat or as an opportunity, those who choose the latter will thrive. This is seen in all facets of life and the process that leads to success is known as evolution. During the last 25 years we have used the evolution of information technology and its developments along with the rapid development of numerous scientific techniques to augment our resources so that we may respond to changes and improve our service to clinicians, patients and thereby our funding organisations.

The road ahead looks exciting with the arrival of innovative technologies such as: intracorporeal monitoring devices, systems that are able to monitor biochemical analytes in patients real-time; artificial intelligence used to markedly improve tumour detection; and virtual reality as a means to speed up rehabilitation. Secure personal internet health portals such as *Manage My Health* have become commonplace and enabled patients to access their medical records and take an active role in their health management. The healthcare and laboratory future will probably involve change at a faster pace than we have seen to date. By carrying forward the experiences of the last 25 years we should be able to meet the challenges we encounter and evolve to meet or exceed the demands made of us in the future.

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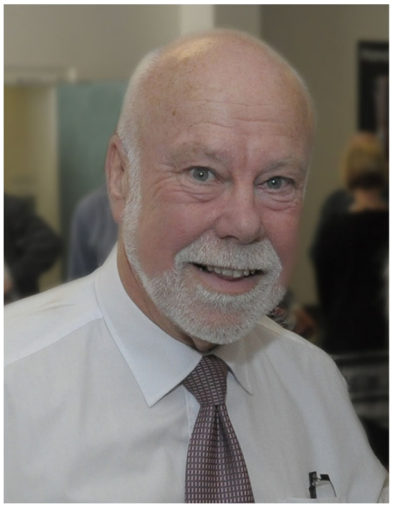
OBITUARY

Richard Ward



Richard emigrated from England with his wife Ann who was a New Zealander. Richard had a BSc(Hons) from the University of Hull in England and therefore sought a role in the laboratory. However, Richard joined the Waikato Health Board as a payroll clerk in the mid 1970s. He joined the laboratory in the Biochemistry Department around 1976 as a "Graduate Technologist". "Graduate Technologist" roles existed as an alternative to the Basic Training Certificate for entry into what is effectively the equivalent of a Medical Laboratory Scientist role. Richard obtained the position of Graded Officer Quality Control and that was the beginning of his interest and contribution to Quality in New Zealand. Richard developed what has now become the Waikato QA programme as initially a means of helping the regional "T" Laboratories (Rural Labs) in the greater Waikato catchment. The programme as it is today is in a large part due to Richard's combined skills in quality and business management.

Richard had a keen curiosity for things scientific and historical and had a broad knowledge covering many fields including horticulture, Roman history, metallurgy, apiculture, and chemistry to name but a few. Richard was a humble genius who led his staff with humour and care. He contributed a huge amount to medical laboratory science and to the laboratory community. Richard had a talent for identifying marketing opportunities and his innovative approach led to rapid expansion of the QA programme which has become well regarded across Australasia.

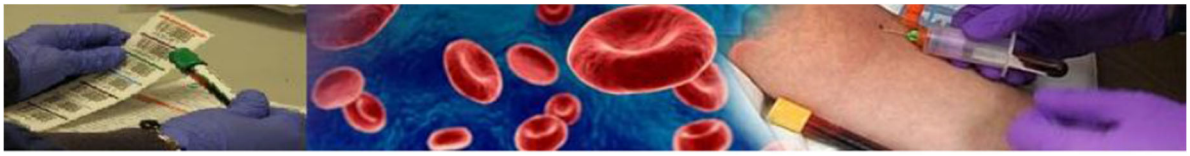
It was with great sadness that we farewelled Richard who passed away in September 2020 after a short illness.

Contributed by Ajesh Joseph

75TH ANNIVERSARY ARTICLE

25 Years in phlebotomy. Aiming for excellence

Annette Bissett and Ailsa Bunker



It's hard to imagine a time when phlebotomy was not recognised as a profession in New Zealand but that was the case 25 years ago. Even the word phlebotomist was not in common use. People that collected blood were generally female and called bleeding lady, blood lady or even vampire. Phlebotomy in most hospital settings was frequently performed by scientists and technicians for an hour or so at the start of their work day, or by laboratory assistants as part of their role.

I (Annette) had been a laboratory assistant at the private lab I worked at for quite a few years, proud of my QTA in General and Biochemistry and happy to do my share of the blood collections before putting on my lab coat to start the bench work. Not a great deal of thought was given to making phlebotomy the focus of my career.

1995, the year before this 25-year story begins, I had left for Papua New Guinea, not to work in the hospitals there, but for a time in a village medical clinic in the heart of the jungle. It was an amazing life-changing experience. I was there partly to be involved with first aid for national people with malaria and machete wounds, and also in a vaccination programme to try stop the spread of a measles outbreak sweeping through the villages.

On returning to New Zealand a few years later I was keen to return to the normal world. I applied for a job as a hospital phlebotomist, thinking it would be good for a while before going back into real lab work. I fell in love with phlebotomy, the hospital environment and my patients. The passion grew. I decided to make this my career and a phlebotomist at North Shore Hospital I have been for the past 21½ years.

Meanwhile on the other side of the city a major part of our phlebotomy history is about to unfold. Did you know that there was no New Zealand qualification in phlebotomy until 2003? In the 1990s I (Ailsa) worked as a part time phlebotomist for 5 ½ years. I saw the frustration of my colleagues to be recognised and valued for doing the same job as me. As a medical laboratory scientist it was easy for me, with my qualification, to say, "Yes" when a patient asked if I was qualified to take their blood. My colleagues could not say the same. I was determined that something needed to be done.

We travelled throughout the hospitals with our handheld plastic tool caddies full of needles and tubes, a holder, a tourniquet, some alcohol wipes, cotton wool balls, roll of tape, and a rather small sharps bin. The caddy was placed on the patient's bed or bedside table. We washed our hands with soap and water between patients, sometimes. We used latex gloves for the messier collects like heel pricks and mycology. There was no way of transferring blood from a syringe to a tube except by using the hypo needle. Lancets could do multiple punctures if you were having difficulty filling sufficient microtainers required for the tests. Samples at 37°C were carried close to the body, usually in the armpit or bra on the walk to the lab. Recapping used needles was what you did to make it safe, if you were out of reach of the sharps bin.

In our world of phlebotomy things needed to change. We needed to take this passion of ours and promote it as a profession, a career, complete with qualification, and standards of practice to follow. In 2001 Middlemore Hospital Laboratory hosted the annual NZIMLS conference. Despite my managers saying, "It can't be done", "We've tried it all before" I convinced them to hold a phlebotomy workshop with the main aim to investigate the establishment of a phlebotomy qualification in New Zealand. I would take responsibility for the day. We had been given a BD discussion document from the UK/Netherlands initiative with a syllabus layout to spark conversation.

It is a day I will never forget. I wanted to get up early to prepare my mind for a successful phlebotomy workshop in the afternoon. The radio was telling news of planes being flown into the World Trade Centre, New York. Yes, it was the 12th of September 2001 or 9/11 in the USA. It certainly was going to be a day in history. How were we going to keep our minds on the job when all this was happening in America? I decided that we would just stow these thoughts away for the duration of the afternoon workshop and keep to our goal to make our own history.

What an amazing day. The organisers were unprepared for the 70+ people that attended this stream of the conference. The space allocated had been underestimated and extra chairs were brought in to accommodate all the people. The time was right. The mood in the room was like a religious crusade. People were ready to make change.

The group wanted a phlebotomy qualification, registration, representation and recognition…even if there was no pay reward. It was the unanimous decision of the delegates that a working party be set up to pursue the development of a qualification in phlebotomy and to promote phlebotomy as a profession. People from all over the country signed up to be part of this group. Teams were established in the main centres and emails were exchanged so that we could communicate and get ideas and consensus.

The working party pursued phlebotomy excellence, qualification, and recognition on four fronts: selecting a qualification model, syllabus, standards, and representation. This work group had a self-imposed deadline to achieve a viable qualification by 2003. We knew that the New Zealand government was discussing registration for medical laboratory technicians and we wanted phlebotomy to be ready with a qualification to support phlebotomists' inclusion.

The qualification: the aspiration was that the qualification was to be national, accessible, affordable, portable, cross- credit to other training programmes, and be of such a calibre as to be recognised internationally. There were several options on the table to consider as a qualification and pathway to registration: The NZIMLS Qualified Technical Assistant (QTA), New Zealand Qualifications Authority (NZQA) unit standards, local qualifications that were both NZQA approved and not, and also overseas qualifications from Australia and USA.

After consideration a pragmatic approach prevailed and the NZIMLS Qualified Technical Assistant (QTA) exam was chosen as a qualification for phlebotomy because it was a well-recognised qualification within pathology laboratories in New Zealand. It was true that it was national, accessible to those employed in the industry, affordable, and portable across New Zealand medical laboratories. However, it was, and is not, on the national qualifications' framework and does not cross credit to other qualifications.

The syllabus: at the initial workshop it was agreed that The BD discussion document was a suitable basis for a syllabus. This was, however, only a start point. The working group donated time and talent to develop a New Zealand phlebotomy syllabus using the best resources available. It is important to note that although we came from both the public and private sectors everyone collaborated generously with their resources. Organisations competitiveness was 'left at the door'. Everyone knew this project was bigger than them and important for phlebotomy and phlebotomists throughout New Zealand. A small working group of people throughout the country met in Wellington one weekend to brainstorm and share details to be included in the syllabus.

The first phlebotomy syllabus was created. Both the Common and Phlebotomy Syllabus were to be part of the phlebotomist's examination with 2yrs/4000hrs experience required to qualify. This was the same structure as other NZIMLS QTA exams. It was important from the start not to be relegated as second rate to other laboratory technicians, hence the alignment to the number of years training experience and the inclusion of the common syllabus. It was also believed and accepted at the time that the examination would be able to cross credit with the other QTAs. At this time if a person held one QTA in an area, if they changed departments they could sit another QTA exam after only one year's extra experience. Sadly this has changed over the years and now this cross credit is not available to pre-analytical staff, even with their exam. Phlebotomy may have its own place imbedded in the industry, however, we can't help but feel there has been an erosion of parity with the separation of pre-analytical technicians from the other medical laboratory technicians by the Medical Sciences Council and the alteration of the examination structure of the NZIMLS with pre analytical specialties separated from other MLTs.

Standards: The National Committee on Clinical Laboratory Standards (now CLSI= Clinical Laboratory Standards Institute) documents were chosen to use as a basis for our own best practice because they were already documents on venepuncture, capillary collects, urine collects and other standards which were updated regularly and a product of international consensus. These are still the core documents for standard operating procedures for phlebotomists.

Representation: remaining a subgroup of the NZIMLS was decided as the NZIMLS was already set up to administer examinations, had a database and web page. The Institute was also well established to promote laboratory issues, and this meant our group could attend to things phlebotomy without having to attend to administration. The upside for the Institute was that its membership, therefore finances, would be improved overnight by all the enthusiastic phlebotomists.

What's in a name? The group debated and chose the name New Zealand Association of Phlebotomy -NZAP, said N-ZAP. This was to be in keeping with phlebotomy groups in other countries. The group did not want to be called a SIG as PIG or PSIG. This did not sound as good to be a PIG (Phlebotomy Interest Group) or other iterations to a group of people that were craving recognition and tired of being the brunt of vampire jokes and the like. Therefore the name NZAP with the slogan *Aiming for Excellence* was chosen. The examination qualification at that time was also deemed important enough to have its own name. Instead of Qualified Technical Assistant – Phlebotomy which would have been the nomenclature at the time, the group chose Qualified Phlebotomy Technician as the title for the first examinations. Thus the QPT was 'born'.

During 2002 we took our newly created syllabus 'on the road' to seminars around the country. The idea was to train the trainers about this so that when the exam was offered the following year everyone was ready. It was a huge success and in the first year there were about 170 candidates!!!

Meanwhile the New Zealand Government's Health Practitioner's Quality Assurance Act came into effect in Sept 2004. Medical laboratory technicians were required to be registered and hold an annual license and phlebotomists were included. Our timing had been perfect. We were told that thanks to us in great part to a lay person on the Board who did not think it was right that a person who was using needles on patients was not licensed. Also, I believe that having a qualification available made it easy to insist.

Some members of the phlebotomy group were managers of both phlebotomy and specimen services teams. The needs and recognition of specimen services was not far from our minds. The specimen reception area of laboratories was growing into its own entity. The staff in the area had previously been made up from technicians from other laboratory areas but the workforce was changing. There were a growing number of staff that entered the area that had no qualification and the work was becoming more complex and specialised. It was with great effort by the specimen services people in the group themselves that created a qualification for specimen services. One person was so passionate about getting a qualification that she could be proud of she helped write the specimen services syllabus and then sat the first exam which was held in 2006. Initially the Qualified Specimen Services Technician (QSST) exam was not given the same professional registration status but common sense finally prevailed in 2016 after much pressure from the NZIMLS to the Medical Sciences Council.

The qualifications changed their name in 2010 to be standardised with the other medical laboratory technician exams to be Medical Laboratory Technician – Phlebotomy and Medical Laboratory Technician – Specimen Services, only to be later separated to Medical Laboratory Pre-Analytical Technician – Phlebotomy and Specimen Services. Since then qualification of Donor Technician has been offered and is a registrable qualification. Although not initially involved with our group in recent years the donor technicians have joined the Pre-Analytical Special Interest Group Committee and contribute to seminars and conferences along with phlebotomy and specimen services.

Meanwhile, back in the wards and collections centres around the country, our work in phlebotomy continued. In 2002 MRSA appeared so focus on contact precautions and hand hygiene was stepped up. The chain of infection was a frequent topic of discussion and universal precautions meant we took more seriously that every sample could be a source of infection. Purell hand sanitisers were introduced so that we could be sure not to become the transmitter of the nasty bug. Latex gloves for blood and non-blood collections were available and we got used to gowning and gloving to protect ourselves and our patients from the many microorganisms that did the rounds through the hospitals and communities. Now everyone is familiar with the WHO 5 Moments of Hand Hygiene and has incorporated them into our standard operating procedures.

Much Information Technology has been introduced into phlebotomy during the last 25 years. Most laboratories now have a system of electronic ordering for laboratory requests which provides a paper form as reference for verbal identification with visual checks against the labels and wristband. The next improvement will be the introduction of portable barcode scanners and label printers as we enter the next phase of paperless e-orders. Hopefully this will be the means of reducing or eliminating the incidences of mislabelled and unlabelled samples. Some laboratories have already supplied their phlebotomists with mobile phones which allow connectivity to the laboratory LIS and access to the laboratory test code manuals for specific collection information and team communications.

Improvements in technology related to the equipment we use have been on-going bringing about improvements in the patient experience, staff safety and sample quality. A big enhancement was Extra Thin Wall technology which increased the internal diameter of the collection needles which improved the flow rate quite significantly. It means that the use of a smaller gauge needle for difficult fragile veins or children was less likely to compromise the sample due to haemolysis. In our DHB we have seen significant reduction in this type of collection error. The sharpness of the bevel has been improved making the incision less painful and many patients comment favourably on it.

Around 2012 safety needles were introduced into many DHBs as routine collection needles. The Eclipse needles with their safety guards to lock over the used needle and the Push Button Winged Sets with their in-vein retractable needle have been great improvements in staff safety. Needle stick injuries, although not frequent, were always at the back of our minds as we worked in some tricky situations to collect blood from our patients and they provide an extra measure of safety for us in situations where the patients are not always cooperative or the sharps bin is not within immediate reach.

Blood collection tubes have also undergone transformation: glass to plastic, haemogard stoppers to reduce splashes on opening, and gel separators to prolong specimen stability. Mechanical separators are now being introduced to improve the quality of plasma samples, shortening the processing time and eliminating some of the problems that have been associated with gel interference with some analytes. Most hospital phlebotomists now operate from a trolley that allows for a full range of collection equipment and consumables to be within arm's reach. Each has a worktop that allows for better infection control measures. Most all equipment is now single-use and disposable.

The extended Scope of Practice of Cannulation has been included in the area of phlebotomy by the Medical Laboratory Sciences Council allowing registration to do this task. This is has been seen as a good thing as phlebotomists certainly have skills that are aligned with this task.

Phlebotomy pre-analytics is now main stream at conferences as well as holding its own workshops. We have been active since the first days of the group with one of the first seminars being organised by the South Island branch in Christchurch holding a memorable event with Dennis Ernst, author of Phlebotomy Today, Founding Director of Center for Phlebotomy Education Inc, and text book author being an invited guest. Other highlights have been being included in and organising the mainstream events such as the NZIMLS Annual Scientific Meetings and the South Pacific Congress in Auckland (2007, 2015) and in Australia (2019). Over the years we have had the pleasure of inviting other renowned international speakers to our conferences including Dr Kathleen Becan McBride - co-author with Diana Garza of the Phlebotomy Handbook, Twyla Rickard - Laboratory Operations Manager for the Clinical Core Laboratory at Mayo Clinic in Minnesota, and Professor Guiseppe Lippi from Italy presented his paper on preanalytical variability (1). Many of our group have been invited to speak at Australian conferences starting with Ailsa's trip to Townsville to speak at the celebration of 100 years of Tropical Medicine Conference 2010, and seven of us speaking at the AIMS Tropical Conference in Whitsundays in 2018.

The pre-analytical area of the laboratory as a whole has gone from strength to strength. We truly have come of age and our contribution to the national and international laboratory scene is recognised. Much has changed and continues to change in our world. Our motto remains the same.:**Aiming for Excellence.**

THE END 

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Comparison of histological staining techniques for copper

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Objectives: The aim of this study was to trial two stains previously unused by Canterbury Health Laboratories Histology Lab (CHL) for copper. An underperforming copper stain was suspended from use at CHL since 2018, due to inconsistent and unreliable results. A standardised and safe stain for copper with reproducible results is required.
Methods: Formalin-fixed, paraffin wax embedded (FFPE) sample tissue, donated by LabPLUS Auckland, was cut using a rotary microtome onto white adhesive slides. Tissue was obtained from an anonymous patient positive for Wilson's disease. Stains trialled included: the original suspended dimethylaminobenzylidene rhodanine stain (modified Lindquist's rhodanine technique, CHL 1990); Victoria Blue technique (Newcomer 2021); and the Abcam Copper Stain Kit (rhodanine technique, Abcam, 2018). Validation of the successful method was carried out using results provided by LabPLUS for 4 positive cases and 4 negative cases of copper accumulation.
Results: The Abcam Kit method showed successful staining that was safe, standardised, reproducible, and validated using patient results supplied by LabPLUS Auckland. Victoria Blue was unsuccessful, only showing diffuse nonspecific staining and introducing more health and safety risks, so was rejected for use at CHL.
Conclusion: This study identified the Abcam Kit as a successful stain to be used by CHL for the investigation of copper accumulation in routine tissue specimens. This allows the diagnosis of patients with Wilson's disease and other hepatic abnormalities and eliminates the need to send specimens to LabPLUS Auckland for investigation of copper accumulation

Evaluation of fresh walnut versus commercial walnut allergen for skin prick testing

Jonathan Cheong[1] and Roy The[2]
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Objectives: Skin prick testing is a test used to aid in the diagnosis of IgE-mediated allergy. We compared the results (wheal and flare) obtained for skin prick testing using crushed fresh walnut as the allergen versus commercial walnut allergen.
Methods: Fresh walnut and commercial walnut allergen skin prick testing were performed on 20 patients with suspected cases of walnut allergy. Patients with dermatographism, or were unable to stop antihistamine medication, were excluded from the test. The wheal and flare were measured (in mm) 15 minutes after skin prick testing were performed.
Results: The fresh walnut had a higher maximum wheal (14mm) than commercial walnut allergen (7mm). The fresh walnut also had a higher mean wheal compared to commercial walnut allergen (7mm and 1.5mm respectively). The correlation coefficient of fresh walnut against commercial walnut allergen was determined to be 0.487, while the coefficient of determination is 0.237. Bland-Altman analysis revealed that there was a bias of +5.5mm, with a standard deviation of 4.03mm, when comparing fresh walnut to commercial walnut allergen. The fresh walnut gave 16 positive results and 4 negative results, while commercial walnut allergen gave 9 positive results and 11 negative results.
Conclusion: The fresh walnut and commercial walnut showed poor correlation in results. Determination of sensitivity and specificity, negative predictive value and positive predictive value may guide the selection of one allergen over the other.

Influence of storage time on stability of routine coagulation parameters (INR, APTT, and fibrinogen) at room temperature

Richard Chen[1], Yii Sen Wee[2] and Rhonda Lucas[2]
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Objectives: Extending the maximum acceptable specimen age for testing/retesting some routine coagulation parameters has many benefits (e.g., reagent evaluation, fibrinogen add-on for DIC, or addressing sample delay during snow in Otago/ Southland). This study assessed such a possibility, where stability of INR, APTT, and fibrinogen results were examined in relation to storage time.
Methods: From each participating individual (50 total), 4 citrate tubes were collected. A baseline tube was centrifuged and tested for INR, APTT, and fibrinogen at time of arrival. The other three tubes were kept as whole blood. After 24h, 48h, and 72h from time of collection, 1 tube was taken for centrifuging and tested for INR, APTT, and fibrinogen. In addition, centrifuged tubes were retested for INR and fibrinogen after 24h, 48h, and 72h (centrifuged fibrinogen after 96h) from time of collection. All specimens were kept at room temperature.
Results: The mean within-individual biological variations of INR, APTT, and fibrinogen at after 24h, 48h, and 72h (centrifuged fibrinogen after 96h) were calculated and compared with specific allowable limits of performance (ALP). For both centrifuged and uncentrifuged specimens, INR variations up to 72h all passed RCPA ALP (±0.3); fibrinogen variations up to 72h (centrifuged fibrinogen up to 96h) all passed EFLM ALP (±10.7%). APTT had a clear increasing trend, and all of its variations failed the EFLM ALP (±2.7%).
Conclusion: Evidence supports the extension of maximum acceptable age of uncentrifuged specimens for INR and fibrinogen tests to 72h. Changing the maximum allowable age of APTT is not recommended. For centrifuged specimens, the acceptable age for INR can be extended to 72h and for fibrinogen, to 96h.

Distinguishing between foreign pigmented lesions of the oral cavity using histological methods

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Objectives: Foreign pigmented lesions are common in the Oral Pathology Centre, often appearing similar. This study aimed to determine a histological staining profile that allows for easier discrimination between the foreign pigmented lesions; Amalgam Tattoo and Melanotic Macule.
Methods: Formalin-fixed, paraffin-embedded wax blocks were collected from patients who were biopsied at the University of Otago Dental School and were diagnosed with an amalgam tattoo or melanotic macule. Ten slides per diagnosis were produced from the biopsy wax blocks. These were stained using Perl's Prussian Blue reaction for ferric iron and five slides per diagnosis were stained using Masson's Fontana method for melanin. Select specimens from each group were stained using automated immunohistochemistry for S-100 and Vimentin. One specimen from each category was taken for scanning electron microscopy investigation. Retrospective data were gathered from the final histopathology reports of all samples to compare the haematoxylin and eosin morphologic characteristics.
Results: The Masson's Fontana stain showed increased positivity in melanotic macules. The Perl's Prussian Blue only stained positively on two samples; therefore, it was shown to have little diagnostic value. Immunohistochemistry for S-100 demonstrated increased positivity in melanotic macules. Vimentin is a marker for melanocytes and mesenchymal cells which both reside in the basal layer, making this difficult to

interpret. The Scanning Electron Microscope made visualizing the amalgam tattoo obvious as it appeared much brighter and more defined. The retrospective data analyzed showed each diagnosis had its own characteristic profile on haematoxylin and eosin staining.
Conclusion: Routine haematoxylin and eosin staining of tissues remains the preferred, most cost-effective method to discriminate between these lesions. However, a pathologist is needed to ensure results are accurate.

The optimisation and validation of NKX3.1 prostate antibody using immunohistochemical techniques

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Objectives: NKX3.1 is a prostate specific androgen-regulated homeobox gene located on chromosome 8p. The loss of function results in the initiating event of prostate carcinogenesis. NKX3.1 is a differentiating antibody used to aid and confirm the diagnosis of prostatic origin in primary and metastatic carcinomas of unknown origin by employing immunohistochemical techniques. The optimisation and validation procedures establish the clinical validity of the antibody as an adjunct tool, ensuring the antibody's performance and characteristics meet acceptable quality limits and standards.
Methods: The NKX3.1 antibody was optimised and validated on the Leica Bond III automated immunohistochemical system facilitating the Bond polymer refine detection kit. The variables of: the antibody dilution; antigen retrieval solution; and the incubation period of the primary antibody determined the staining result. The optimisation protocol was run on a positive control, a negative control, and a patient prostate chip biopsy.
Results: Of the 10 protocols tested, the 1:100 dilution factor in combination with ethylenediamine tetraacetic acid (EDTA), pH 9 based antigen retrieval solution and an incubation period of 30 minutes at 100°C yielded the best results. This combination produced a strong, nuclear staining intensity in cells expressing the antigen of interest. In addition, the positive and negative controls proved the protocol to be valid.
Conclusion: The validation of the performance parameters proved that the optimisation of the NKX3.1 antibody produced a precise, accurate, specific, and reproducible result. However, further testing of the sensitivity of the antibody is required to develop a protocol that is optimal and validated, providing scientists and pathologists confidence within the testing procedures and the results obtained .

Evaluation of potential carry over risk in the misdiagnosis of tumour markers, Ca 19-9 and hCG on the Abbott Alinity and COBAS-8000 analysers

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Objectives: In the diagnostic laboratory, sequential analysis of specimens occurs routinely. There is potential for contamination by previous specimens when analysing in sequence, particularly if the same sample probe is used. Diagnostic analysers use specific mechanisms to reduce carryover risk, including instrument flags and specific probe washes to limit carryover altering results. Tumour markers such as Ca 19-9 and hCG are more prone to carryover risk, and therefore clinical errors and misdiagnosis, due to the large concentration range and low clinical detection levels. The efficacy of probe wash stations is currently unknown in the Dunedin laboratory and has not been well characterised in the literature. This study aimed to report the effectiveness of probe wash stations on the Abbott Alinity and Roche COBAS-8000 analysers, using previously characterised tumour markers Ca 19-9 and hCG.
Methods: Clinically positive Ca 19-9 and hCG patient samples were run on specified analysers followed by negative patient samples for the tumour marker of interest. A deliberate contamination step was performed, and sequential testing was rerun, with initial and final values noted.
Results: The results indicated minimal carry over. Any change to results did not alter the overall diagnosis. hCG yielded clinically insignificant results. The P-value for hCG was undetermined as the difference between results was zero. The P-value for Ca 19-9 (0.0007^{1}) indicated statistically significant findings that are not clinically significant when compared with the RCPA Analytical Performance Specifications.
Conclusion: This study revealed the probe wash mechanisms on the Abbott Alinity and Roche COBAS-8000 analysers were sufficient in preventing contamination and therefore adequate for producing reliable clinical results.

Evaluation of need for sample preincubation in rheumatoid factor analysis

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Objectives: At Canterbury Health Laboratories, samples are currently preincubated at 37°C before rheumatoid factor (RhF) analysis. This is a historical step thought to prevent potential false rheumatoid factor concentration results caused by precipitation of cold-reactive rheumatoid factors, found in conditions such as mixed cryoglobulinaemia, due to lower sample temperature. This study was performed to determine the necessity of sample preincubation in RhF analysis.
Methods: Serum samples (116), with known detectable RhF, had RhF concentrations measured using endpoint nephelometry with a Siemens BNII Nephelometer and Siemens N Latex RF Kit. Samples were first analysed after thawing at 4° C, then reanalysed after incubation at 37°C for an hour. The significance of the difference between RhF results was determined using the Royal College of Pathologists Australasia's (RCPA) analytical performance specifications (±12 difference for less than 60IU/mL, ±20.0% for 60IU/mL or more). MedCalc statistical software was used for Passing-Bablok regression and Bland-Altman statistical analysis.
Results: Only two patient samples (1.72%) showed significant measured RhF concentration variation between methods. These patients both had Sjögren's syndrome antibodies. Bland-Altman plot limits of agreement (LoA) and 95% confidence intervals (CI) were within RCPA specifications. LoA for samples measuring less than 60 IU/mL: -9.77 IU/mL (CI: -11.90 to -7.64 IU/mL) and 5.58 IU/mL (CI: 3.45 to 7.71 IU/mL). LoA for samples measuring 60 IU/mL or more: -13.48% (CI: -15.69 to -11.27%) and 8.49% (CI: 6.28 to 10.71%). Passing-Bablok showed no significant systematic or proportional difference between methods (intercept: -0.7419 (CI: -2.0 to 0.1684); slope: 0.9901 (CI: 0.9717 to 1.0)).
Conclusion: This study suggests that sample preincubation for RhF nephelometric analysis may be discontinued to reduce RhF testing time. Further investigation into RhF analysis for patients with Sjögren's syndrome is recommended.

Verifying a Radiometer® Hemocue WBC Diff device

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Objectives: The Hemocue WBC Diff is a point of care device (POC) that provides a total leukocyte count (WBC) and a 5-point differential count of neutrophils, lymphocytes, monocytes, eosinophils, and basophils. This verification study was performed to verify a Hemocue WBC Diff's fitness for purpose within the scope of its intended use, as required by the SCL Quality Assurance program. The reference method for this study was the Sysmex XN 2000 automated haematological analyser.

Methods: To verify this device 50 venous samples were selected based on the following criteria: (1) white cell total count, (2) lack of abnormal cell flags, and (3) time elapsed since sample collection. WBC count values were between 1-30x10^9/L. Samples were measured by the Hemocue within 4 hours of sample collection. Blood films of the samples were examined to identify possible morphological interferences.
Results: Bias for WBC, neutrophils, and lymphocyte counts were -0.610x10^9/L, -0.576x10^9/L, and 0.162x10^9/L. These results were statistically significant; p-values were ≤0.05.
Precision values for low and high precision check samples were within the Royal College of Pathologists Australasia (RCPA) quality assurance programme (QAP) analytical performance specifications (APS) for Hemocue POC devices.
WBC, neutrophil, and lymphocyte counts showed strong correlation with the reference method. Adjusted R^2 values of 0.995, 0.992, and 0.903 for WBC, neutrophil, and lymphocyte counts were obtained.
Monocytes, eosinophils, and basophil counts had CV values exceeding 20%, indicating poor precision.
Conclusion: This Hemocue WBC Diff POC device provides clinically relevant and accurate total WBC, neutrophil and lymphocyte counts in the context of acute medical care. In the absence of a differential count being generated accurate WBC counts can still be obtained. Confirmation with automated haematological analysis is recommended.

Evaluation of the effect of high-speed centrifugation on haemostasis specimens using the Heraeus Pico 17 centrifuge and the Stago STart Max analyser

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Objectives: Increased sample turbidity interferes with optical clot detection on the Sysmex CS-5100 (Siemens) automated analyser. One method to remove turbidity is high-speed centrifugation of the plasma at 17,000g for five minutes and re-analysis of the infranatant. If there is interference with the optical method after high-speed centrifugation, the STart Max (Stago) provides an alternative mechanical clot detection method. Previous in-house evaluation of high-speed centrifugation found the Activated Partial Thromboplastin Time (APTT) was shorted by up to 2.4s (mean value 1.0s) with re-analysis on the CS-5100 (Siemens). This study aimed to evaluate the effect of high-speed centrifugation on Prothrombin Time (PT), International Normalised Ratio (INR), APTT and fibrinogen results when re-analysed on the STart Max (Stago).
Methods: Sodium citrate samples were initially centrifuged at 3195g for five minutes in the Thermo Scientific Megafuge 16 (Thermo Fisher). An aliquot of each sample was then centrifuged at 17,000g for five minutes in the Heraeus Pico 17 high-speed centrifuge (Thermo Fisher). Plasma centrifuged at each speed was then tested for PT/INR (*n=40*) for APTT (*n=40*) and fibrinogen (*n=20*) on the STart Max (Stago) analyser and results were compared. The laboratory Measurement of Uncertainty (MU) was used in assessing the results, as well as statistical analysis performed using Bland-Altman plot, Passing-Bablok regression.
Results: In comparison of results from 3195g and 17,000g centrifuged plasmas, PT results showed a mean bias of -0.7s, INR a mean bias of -0.05 and fibrinogen a mean bias of +0.13g/L, which are not statistically significant differences. However, APTT results showed a statistically significant mean bias of -3.0s, with differences in results up to 8.7s. 50% were outside the APTT MU (8.7%).
Conclusion: This study found the effect of high-speed centrifugation on STart Max (Stago) APTT results to be significant, and is greater than the previous CS-5100 (Siemens) study. There was no significant difference in PT, INR and

The future of Rubella IgM testing at Canterbury Health Laboratories

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Objectives: This report aimed to identify whether the Euroimmun Anti-Rubella Virus Glycoprotein ELISA (IgM) kit is an acceptable replacement for the Siemens Anti-Rubella-Virus/IgM kit (World Health Organisation recommended) which stopped production at the end of 2020. The World Health Organisation is yet to recommend a new kit for Rubella IgM testing. Rubella is a viral infection spread through respiratory droplets, producing a speckled rash on patients. It has an incubation period of two to three weeks prior to symptoms (rash, fever, headache, enlarged lymph nodes, etc.) after which anti-rubella IgM is detectable.
Methods: Stored samples previously tested at Canterbury Health Laboratories using the Siemens Anti-Rubella-Virus/IgM kit were retested using the new Euroimmun kit. Other samples from The Royal College of Pathologists of Australasia were also tested on the Euroimmun kit and compared to results from numerous laboratories involved (using different methodologies) throughout Australasia as a form of external kit validation. Cross-reactive agents specified on the Euroimmun kit insert were also tested to identify any kit limitations.
Results: The results from this research project demonstrated that the Euroimmun kit sufficiently reports results comparable to the World Health Organisation recommended Siemens kit (and other kits used across Australasia) while suspected cross-reactive agents do not appear to interfere with results. Besides reporting, the efficiency, comprehensibility and accessibility of the Euroimmun kit make it a step up from the Siemens kit rather than just a substitute.
Conclusion: It can be concluded the Euroimmun Anti-Rubella Virus Glycoprotein ELISA (IgM) kit is an acceptable replacement for the Siemens Anti-Rubella-Virus/IgM kit, with greater sensitivity and specificity essential for diagnostic use.

Expanding the range of tests for the Stago STart Max coagulation analyser and standard operating procedure production

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Objectives: The Wairau hospital laboratory has a Stago STart Max point of care coagulation device. The STart Max software is difficult to navigate and use but it is a useful backup analyser to the Sysmex CS2500. The primary aim of this project was to calibrate the fibrinogen assay on the STart Max and ensure the assay was clinically viable. The secondary aim was to ensure the STart Max thrombin clotting time assay was operational and clinically viable. The tertiary aim was to produce standard operating procedure for these two STart Max assays, as well as the STart Max prothrombin time and activated partial thrombin time, which were created and handwritten prior to this project.
Methods: A calibration curve was produced for the fibrinogen assay and the process recorded for reproducibility. The thrombin clotting time, not requiring a calibration, was made available on the list of tests on the STart Max, allowing it to be used. Thirty sample comparisons were performed for both assays on the STart Max and the CS2500. The thrombin clotting time required an additional 16 sample comparisons after statistical analysis. Standard operating procedure was typed up and refined with supervisor feedback.
Results: The 30-sample comparison for the fibrinogen assays produced a correlation coefficient of 0.990 and a regression graph demonstrating a slight positive bias. The thrombin clotting time had an initial correlation coefficient of 0.780 but, with the

addition of 16 more samples, which provided a broader range of results, the correlation coefficient became 0.954. There was no significant bias in the thrombin clotting time. The standard operating procedures were adequate.
Conclusion: The Wairau hospital laboratory has clinically viable backup methods for fibrinogen and thrombin clotting time assays. They also have clear and easy to follow standard operating procedure for each of their STart Max assays.

An evaluation of the Hemosure Accureader A100 *Helicobacter pylori* assay

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Objectives: The Hemosure Accureader A100 is a colorimetric analyser now available for use in New Zealand clinical laboratories. This report evaluates this analyser, specifically the *Helicobacter pylori* assay. This assay was evaluated and contrasted against the CerTest *Helicobacter pylori* card-based assay; a highly accurate testing kit currently used by multiple laboratories in New Zealand.
Methods: Seventy faecal samples were selected during standard laboratory function and run through both testing methods to compare results. Inconclusive results were retested. Samples with repeated discrepant results were sent to Dunedin SCL to be tested on the Liaison XL immunological analyser as a confirmatory test. Both CerTest and Hemosure utilise latex immunochromatography based lateral flow assays to provide results.
Results: Testing revealed the CerTest assay was more accurate, specific, and had a higher positive predictive value than the Hemosure assay (91.4%, 95.6%, and 90.9% against 68.5%, 56.5%, and 52.4% respectively). For twenty samples sent for confirmatory testing, CerTest presented with higher accuracy against Hemosure (85% and 10% respectively). From the confirmatory testing, Hemosure presents with a high rate of false positives with values being reported from 61ng/ml to 257ng/ml, however this may be limited due to the analysers' adjustable cut-off values.
Conclusion: Due to the low accuracy, the suboptimal positive predictive value, and the high rate of false positives, the Hemosure Accureader A100 *H. pylori* assay cannot be recommended for use inside Wellington SCL at this time. Further advice is needed from the manufacturer to improve the performance of this test at Wellington SCL.

Is the BioMérieux ASD-Kit a suitable replacement for the Beckman Coulter IMMAGE in the determination of anti-streptodornase B in test serum?

Carne Lincoln
University of Otago, Dunedin

Objectives: To determine if the BioMérieux ASD-Kit is a suitable replacement for the Beckman Coulter IMMAGE for the detection of anti-streptodornase B in test serum.
Methods: Through the application of the BioMérieux ASD-Kit, semi-quantitative analysis of test serum determined the titre of anti-streptodornase present in serum and therefore the clinical significance of this titre. The test utilises a single dilution of test serum and increasing quantities of Streptodornase B in a strip of wells. Operating under the principle of inhibition, a dye colour change from blue to pink illustrates the absence of anti-streptodornase B in test serum and therefore the inability to neutralise streptodornase B .
Results: Patient samples with known clinically significant anti-streptodornase titres and quality control samples, with semi-quantitative titres previously determined by BioMérieux ASD-Kit, were analysed. Patient samples with a previous clinically significant titre of anti-streptodornase B, indicating current or recent Group A Streptococcal infection, were determined using the BioMérieux ASD-Kit to yield a clinically significant titre at a 100% success rate. Furthermore, 100% of previously negative patient samples were determined as having clinically insignificant titres of anti-streptodornase B indicative of no current, or past, Group A streptococcal infection. Additionally, 100% of quality control samples provided by The Royal College of Pathologists of Australasia and Waikato District Health Board Quality Assurance Programmes yielded titre results identical to their prior established values.
Conclusion: The BioMérieux ASD-Kit has been established as a suitable replacement for the Beckman Coulter IMMAGE in the determination of anti-streptodornase B antibody titre and therefore the clinical significance of antibody titre.
Acknowledgement: The author thanks staff at Waikato District Health Board Laboratories, Hamilton for supervision of this project.

Establishing key performance indicators for faecal pathogen PCR and BRAF mutation assays

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Objectives: Key performance indicators (KPIs) are objective measures for quality assurance and performance monitoring within the diagnostic laboratory and are required as part of the IANZ ISO15189 standard. The aim of this study was to establish meaningful key performance indicators for the turnaround times of faecal pathogen PCR and BRAF mutation assays.
Methods: All faecal pathogen PCR requests from the 01/01/2019 to the 31/03/2021 and all BRAF mutation requests, from the start of testing (July 2019) to 31/12/2020, were extracted from the laboratory information system. This data was used to determine the turnaround time KPI for faecal pathogen PCR and BRAF mutation assay results. Turnaround time targets were developed based on clinical performance and contractual requirements. Performance acceptance criteria were determined based on known operational procedures and workflow.
Results: Two KPIs for faecal PCR testing were established. A clinical KPI to report 95% of results within 3 days and operational KPI to report 97% of results within 4 days. For BRAF mutation testing one KPI was determined with the aim to report 90% of results within 3 days was set.
Conclusion: Turnaround time KPIs were developed for the faecal pathogen PCR and BRAF mutation assays. These key performance indicators are now able to be used by the Molecular Pathology department to continually monitor performance.

Comparison of an immunochromatographic test with a chemiluminescence immunoassay on faecal samples for the detection of *Helicobacter pylori* antigen in active infection

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Objectives: *Helicobacter pylori* (*H. pylori*) causes gastritis, peptic ulcers and is associated with gastric adenocarcinoma. Accurate diagnosis and efficient treatment can eliminate *H. pylori*, resolving these conditions and reducing cancer risk. SCL Dunedin intends to adopt the LIAISON® chemiluminescence immunoassay (CLIA) to diagnose an *H. pylori* infection.
Methods: The diagnostic accuracy of the CLIA was determined using the CerTest immunochromatographic lateral flow test as reference standard. The results from the CerTest are subjective; antigen presence initiates a colorimetric reaction, indicating a positive result. These results were reported on the

ULTRA database and used for comparison. Forty-two patients with symptomatic queried *H. pylori* infection provided stool samples. These samples had been tested by the CerTest and subsequently frozen. These were defrosted to room temperature and faecal *H. pylori* antigen was quantified by chemiluminescent immunoassay, examined on the Diasorin XL LIAISON® Analyser. Antigen concentration is proportional to relative light units which are converted to an index value. Index values range from 0.01 to 50; <0.90 is negative, 0.90-1.10 is equivocal and >1.10 is positive. Study results were reported as qualitative results for comparison to the CerTest.
Results: There was 67% concordance and a moderate agreement between the LIAISON® and CerTest (κ = 0.50). The LIAISON® achieved a sensitivity of 59.1% and specificity of 78.9% when compared to the CerTest.
Conclusion: This study showed poor agreement between the LIAISON® and CerTest. This may reflect methodical issues, subjective CerTest interpretation or sample issues. Additional analysis is required before the LIAISON® can be adopted as an *H. pylori* diagnostic method in SCL.

The validation of the laboratory-developed human adenovirus real-time PCR on the BD Max platform

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Objectives: The objective of this study was to validate a real-time PCR assay on the BD Max platform for detecting human adenovirus DNA in combination with a sample process control based on the modification of the current in-house assay.
Methods: The validation of the BD Max system was compared to the in-house assay
used in Canterbury Health Laboratories. The fully automated BD Max system combines DNA extraction and PCR amplification for improved workflow and reduced labour requirements preventing many of the manual errors associated with PCR. The current in-house assay for detecting human Adenovirus involves a three-step process, where the DNA extraction is completed on the NucliSENS EasyMag instrument, and the Light Cycler 480 instrument achieves PCR amplification.
Results: All samples from the 2020 external proficiency panel were correctly identified by the BD Max system. For the clinical validation, 39 specimen samples from specimen types such as nasopharyngeal swabs, nasal swabs and eye swabs were tested. These specimen samples had been previously analysed with either the in-house assay or the respiratory multiplex commercial assay for the detection of human Adenovirus. From these samples, one presumptively positive sample was undetected by the BD Max system and the in-house assay when retested, all negative samples were correctly identified as undetected by the BD Max system. The BD Max system detected the PCR signals 2 to 7 crossing threshold (Ct) values earlier than the in-house assay or the respiratory multiplex assay.
Conclusion: The fully automated BD Max assay showed improved workflow with superior analytical sensitivity and an excellent detection of human Adenovirus in the various specimen types.

Pre-analytical time delay of five hours on platelet function as measured by PFA-200

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Objectives: Platelet function analyser (PFA) provides a simple, rapid, qualitative and precise automated assessment of platelet adhesion and aggregation as it assesses for any inherited, acquired, or drug induced platelet dysfunction, especially von Willebrand disease. It is also used to monitor desmopressin therapy in both, and possibly anti-platelet therapy. The aim of this study was to investigate platelet function by comparing the closure times of within 1 hour and 5 hours after sampling to see if the pre-analytical time delay influenced platelet function. This will enable us to formulate a possible extension for our PFA turnaround time.
Methods: Citrate tubes from 20 participants were delivered immediately (within the first hour in the laboratory) for analysis using a PFA-200 microprocessor-controlled unit by measuring the closure time (CT) in seconds. Each donor samples was re-processed after five hours for a data comparison to see if there was a significant change under the influence of pre-analytical delay.
Results: The mean CT in two different time points for both agonists demonstrated a significant difference as it showed a 20% and 22% increase for both epinephrine and ADP respectively after 5 hours. The CT of the two different time points correlated positively in both agonists. The mean of the 20 samples in 1 hour and after 5 hours after sampling for both agonists reached significance ($p<0.05$).
Conclusion: There was a systematic and proportional difference between the two means of the different time points in both epinephrine and ADP. The pre-analytical delay of 5 hours does affect platelet adhesion and aggregation and therefore we can exclude the idea of extending the PFA turn-around time PFA-200 for Waikato DHB.

Comparison of 37°C indirect antiglobulin test incubation times in tube

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Objectives: The New Zealand Blood Service currently performs routine tube indirect antiglobulin tests (IAT) using a standard 60 min 37°C incubation time. However, the method recommended by Lorne Laboratories Ltd for their anti-human globulin reagent suggests a 15 min incubation time. Introduction of a shorter method would be of significant benefit due to the decrease in time taken to process samples. The aim of this project was to investigate the significance of reduced incubation times in terms of the resulting reaction strengths. It also acted as a preliminary test for the validation process that is required for the implementation of a shorter IAT incubation time.
Methods: Three quality control specimens with known red cell antibodies were used to perform antibody screens using the tube IAT technique. Each specimen was tested with three different 37°C incubation times – 15 min, 30 min, 60 min. The presence of agglutination was measured optically according to standard tube technique grading and reaction strengths were recorded.
Results: Positive reactions were observed for each incubation time length, as was expected according to the known red cell antibodies of the specimens and known screen cell phenotypes. For each specimen, the 30 min incubation resulted in the strongest reaction strength when compared to the grades of both the 15 min and 60 min incubations. **Conclusion:** The results suggest that the optimal tube IAT incubation time is 30 min. However, a more extensive investigation would be required to confirm these findings. A 30 min incubation time should be included in the official validation process .

A comparative analysis of the Sysmex XN-20 to the manual microscopy method for schistocyte counts

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Objectives: Schistocytes are the product of red cell fragmentation and important in the diagnosis of microangiopathic haemolytic anaemia. At Canterbury Health

Laboratories, scientists will perform manual schistocyte counts on a sample, when requested by a clinician. The aim of this study was to determine if the Sysmex XN-20 can replace the need for a manual count by comparing automated counts with manual counts performed by scientists of varying levels of experience.
Methods: 20 venous blood samples positive for schistocytes were analysed by the XN-20 over a 2 month period. A blood film created from each sample was analysed by three scientists (expert, competent and trainee) manually via microscopy. Analyser counts were compared to the expert's results, which were considered to be the gold standard.
Results: The results of the Sysmex XN-20 and scientists were compared using linear regression and achieved coefficients of determination of 0.18, 0.98 and 0.99, respectively. Bland-Altman difference plots were produced from the same data set, demonstrating a bias of 0.99 between the XN-20 and expert scientist. The competent and trainee scientists demonstrated a bias of 0.90 and 1.9, respectively, in comparison to the expert scientist. Reproducibility tests demonstrated a coefficient of variation of 38.4% for the Sysmex XN-20 and the expert, competent and trainee scientist achieving 21.3%, 29.2% and 18.6%, respectively.
Conclusion: The analyser was found to poorly correlate with the gold standard and demonstrated erroneous results. Reproducibility was also found to be poor, thus confirming that the analyser should not be used to replace manual methods. The manual counts, despite the level of experience, demonstrated a high correlation with the expert, however demonstrated low reproducibility.

A comparison between two Siemens' thrombin clotting time (TCT) reagents: Test Thrombin and Thromboclotin®

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Objectives: The aim of the study was to compare the Test Thrombin reagent, which is currently used for TCT at CSCL, with another TCT reagent, Thromboclotin®.
Methods: To test different aspects of the two reagents, 136 platelet-poor plasma samples (PPP) with a wide range of clotting times were selected. A number of these samples were diluted to achieve low fibrinogen levels (i.e. <1.0 g/L). TCT was performed using both reagents, results were statistically analysed using Analyse-It, and sensitivity and specificity were calculated for Thromboclotin®. Additionally, the precision and reference interval provided by the manufacturer were validated. Sample cost was calculated and compared for both reagents.
Results: There was 87.5% consistency of result interpretation between the two reagents. Despite some discrepancy, in 12.5% of the samples tested, statistical analysis showed no significant overall difference between the two reagents. Most of the discrepancy occurred in samples with very low fibrinogen, as Test Thrombin appears to be more sensitive in detecting low fibrinogen concentrations in PPP samples. In this study, precision was calculated for Test Thrombin to be 0.72%, compared to 1.9% by the manufacturer, while it was 0.86% for Thromboclotin®, compared to 1.3% by the manufacturer. Thromboclotin® had 62.5% sensitivity and 95.2% specificity compared to Test Thrombin. Thromboclotin® is slightly cheaper, but if other local factors are taken into consideration, e.g. reagent storing, aliquoting, and possible wastage, then the Test Thrombin will be overall more economic to use under CSCL's conditions.
Conclusion: There is no significant difference between the two reagents, however, the Test Thrombin appears to be more suitable and practical to use at CSCL, considering its local conditions.

Mechanical versus optical INR detection: A comparative investigation

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Objectives: Both mechanical and optical methods are used to determine prothrombin time, however these methods have different reference ranges and thus the unitless ratio INR is necessary to compare results across methods. The INR should be consistent across these methods. However, this was not the case for a sample analysed by the Stago CM2 and Sysmex CS2500 analysers used in Taranaki labs. Thus, this investigation was carried out to assess analyser agreement.
Methods: Twenty-five samples were run on both analysers to obtain INR values for comparison. The samples gathered over a four-week period to obtain a wide range of INR values and were frozen together until analysis to maintain viability. The Sysmex analyser utilised Dade Innovin reagent, and the Stago utilised Stago INR Neoptimal 10 reagent. Both analysers passed QC checks and were up to date with maintenance and calibration prior to analysis.
Results: The two INR results showed strong correlation (r=0.9772) but the Stago analyser was observed to have a mean difference of -0.17 compared to the Sysmex analyser. The 95% limits of agreement for the Stago analyser were broad: the upper limit 0.93 above and the lower limit 1.27 below the Sysmex. Adjusted for an increasing difference with magnitude the limits of agreement for the Stago compared to the Sysmex analyser were between 22% lower and 20% higher results.
Conclusion: Due to the broad 95% limits of agreement, it is implied that these two analysers may not agree sufficiently to be used interchangeably. This is because an INR value variation of up to 20% above or 22% below during monitoring may be significant enough to result in erroneous alteration of patient coagulative treatment.

Evaluation of the criteria for the identification of beta-haemolytic *streptococci* Lancefield groups A, C and G

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Objectives: Group A *streptococci* (GAS) are an important cause of bacterial pharyngitis and a range of skin infections. Severity of GAS infections ranges from mild to life threatening invasive infection. Group C and G *streptococci* (GCS/GGS) are genetically similar to GAS, but clinical significance of isolation is debated. Rapid and accurate identification of b-haemolytic streptococci (BHS) is important, especially in sterile sites. This study aimed to determine an effective criterion that demonstrates reliable species identification of BHS using matrix -assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS), Lancefield latex grouping and the PYR test.
Methods: A total of eighty-five BHS isolates were obtained and identified using the Bruker MALDI TOF MS. All isolates were then Lancefield grouped against all three groups (A, C and G) using the ProLab Diagnostics Prolex Streptococcal Grouping Latex Kit. All Isolates were also tested for PYR enzyme activity using the Key Scientific Products PYR test kit.
Results: MALDI TOF MS correctly identified eighty-three/eighty -five (97.6%) BHS isolates. Two discrepancies were seen among isolates with a spectral score of <2.00. Of the isolates that received a spectral score of ≥ 2.00, Eighty/eighty (100%) were correctly identified. Lancefield latex grouping correctly identified eighty-two/eighty-five (96.5%) BHS isolates, with three discrepancies seen. The PYR test correctly identified

100% of isolates, all forty-four GAS isolates were PYR positive and all forty-one GCS/GGS were PYR negative.
Conclusion: MALDI TOF MS proved to be the most effective method for identification of GAS and GCS/GGS. The current spectral score accepted for reliable MALDI TOF MS identification of ≥ 2.30 is effective, but over half of isolates would require additional latex grouping. Lowering the acceptable score for reliable identification would maintain accuracy of identification and reduce the need for additional latex grouping, in turn reducing laboratory costs.

Developing a SNP profiling panel for the tracking of whole-exome sequencing studies

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Objectives: Single nucleotide polymorphism (SNP) panels that uniquely identify samples are useful for genetic research. Previously developed SNP panels are composed of a high number of SNPs that target mostly intragenic regions. With increasing interest in whole-exome sequencing (WES) at Canterbury Health Laboratories, we aimed to develop a practical, rapid and inexpensive method for genotyping eight SNPs in unknown DNA samples for identity verification.
Methods: A panel of eight SNPs had been identified which exhibit a minor allele frequency of approximately 50%, and a selection of random samples were genotyped for these SNPs using three different methods; Sanger sequencing, high resolution melting (HRM) analysis and ABI SNaPshot multiplex reaction kit. Genotype data was extracted from exome sequences using Agilent Alissa Interpret software. The probability that any individual of European descent would match another was calculated to be 0.4%, assuming no family relations.
Results: A panel of eight SNPs was not sufficient to differentiate two random samples, as two genotypes of the ten samples tested were an exact match; more SNPs are required to achieve sufficient discriminatory power. The ABI SNaPshot multiplex was the most efficient method for genotyping and Agilent Alissa Interpret software was useful in genotyping SNPs in WES data. Sanger sequencing was the most reliable method, while HRM only worked for 1 of the SNPs.
Conclusion: A SNP panel provides a simple, yet powerful, method for the assignment of highly discriminatory identifiers to genetic samples. However, minimising the number of SNPs used while still allowing sufficient redundancy requires further investigation.

Optimization of kappa and lambda light chains on the Ventana Benchmark ULTRA

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Objectives: All immunoglobulins are made up from two identical heavy chains and two identical light chains. Heavy chains define the immunoglobulin class and subclass (IgG, IgM, IgA, IgE and IgD). While the light chains can either be kappa or lambda, the distribution of kappa and lambda differs for different classes of immunoglobulins. In immunohistochemistry, kappa and lambda are B cell specific antibodies used for plasma cell identification and investigation of lymphoid cell related clonality/ neoplasia in a tissue. However, studies have shown that kappa and lambda assay results are challenging due to their low pass rate and low optimal score rate. Thus, the aim of this project was to optimize the kappa and lambda immunohistochemical protocol to produce optimal staining results on lymphomas.
Methods: Kappa and lambda polyclonal antibodies from Dako were used for this project. Five factors of the immunohistochemical protocol were considered for optimization. These were the Ventana detection kit (Ultraview DAB and Optiview DAB), the antibody dilution, the pH of the antibody retrieval solution (CC1 or CC2), the time of antibody retrieval (HIER) and the antibody incubation time.
Results: The original protocol used a Optiview DAB detection kit, an antibody dilution of 1:8000, CC1 solution for 32 min and an incubation time of 40 min. Following the research project, the optimized protocol consisted of an Ultraview DAB detection kit, an antibody dilution of 1:10000, CC1 solution for 20 min and the incubation time dropped to 24 min.
Conclusion: Optimization was through modifications of detection kits, antibody dilutions, incubation time, pH solution and retrieval time. As a result, a pathologist validated diagnostic test protocol was achieved.

Investigating the correlation and agreement of a newly installed coagulation analyser

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Objectives: Medlab Central uses two coagulation analysers interchangeably to produce patient results that have clinical implications. The newly installed analyser had to show adequate correlation and agreement with the pre-existing analyser before it could be used routinely.
Methods: Citrated whole blood samples of both in- and out-patients were used to produce a pair of results for statistical investigation using correlation coefficient and Bland-Altman plots.
Results: The correlation coefficients for INR, APTT, Fibrinogen, and D-dimer were 1.00, 1.00, 0.99, and 1.00 respectively. The Bland-Altman plots showed acceptable agreement within the clinically relevant ranges for all assays tested. Greater difference was seen in the clinically insignificant ranges. The INR showed good agreement with minimal difference of ±0.1 in the range of 0.8-1.8. A positive bias was noted in the INR as the value increased, differing by no more than 0.4 and no less than 0.1. The new analyser showed a negative bias for APTT with most results differing by ± 1 second in the range of 18-50 seconds. At extremely prolonged APTTs, the difference was greater but insignificant. Fibrinogen showed good agreement in the range of 0-2 g/L. The D-dimer assay showed good agreement with the same conclusions drawn 24/26 times.
Conclusion: Clinical relevance had to be considered when interpreting the results. The INR, APTT, Fibrinogen, and D-dimer assay showed good correlation and agreement within their clinically relevant range. Further improvements include subdivision of samples into physiologically- matched groups, collection of more data covering the D-dimer range of 475-525 ug/L, the inclusion of frozen/thawed samples, the inclusion of haemolysed, lipemic, and icteric samples to assess the effect of interfering substances and investigation into all available parameters.

A comparison of Columbia and Tryptic-Soy Base Sheep Blood Agar, and CHROMagar™ StrepA Agar for detection and isolation of β-haemolytic *Streptococcus* species

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Objectives: This project aimed to compare the haemolytic properties of β-haemolytic streptococci (BHS) on Sheep Blood Agar with Columbia Base (CB) against a Tryptic Soy Base (TSB). This project also determined the effectiveness of CHROMagar™ StrepA, a chromogenic agar designed for screening of Group A streptococci (GAS).
Methods: One hundred and eleven patients' throat swabs were streaked onto one CB, one TSB, and one half of a CHROMagar™ StrepA plate each (all media from Fort Richards

Laboratories). The plates were incubated at 35°C with 5% CO_2 for 2 days. The plates were observed after one and two days of incubation. Growth characteristics, morphology, and the appearance/size of haemolysis were compared. Suspect pathogens were identified by catalase testing, Lancefield latex agglutination, or MALDI-TOF MS.
Results: Of the 111 throat swabs, 26 contained BHS, or a member of the *Streptococcus milleri* group. Differences in growth, morphology and/or haemolysis were observed among 19/26. In 7/26, BHS were identified on the TSB plate before the CB plate. Only 1/26 was identified on CB before TSB. Three swabs containing a *S. milleri* group species were not isolated on the CB plate. Of the 12 swabs that contained GAS, it took 2 days for red colonies to appear on the CHROMagar™ plate for 3/12, and 1 day for 6/12. No red colonies appeared for 3/12 swabs.
Conclusion: β-haemolysis is more evident on TSB than on CB and may allow for faster identification of throat pathogens on the former. CHROMagar™ StrepA does not reduce the turn-around-time of results and therefore, does not allow for identification of GAS within one day of incubation.

Method comparison between tube and microcolumn techniques for the indirect antiglobulin test

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Objectives: In routine blood bank serology, antibody identification is more commonly performed through an indirect antiglobulin test using commercial microcolumn cards. However, tube techniques, including the tube indirect antiglobulin test, still remains the "gold standard" for serological testing. This study was done to compare the sensitivities of the two methods.
Methods: Plasma samples were taken from a total of 10 patients that had a positive red cell antibody screen during their Group and Screen tests. Each sample was tested against a set of panel cells (Grifols Perfect Panel 11) twice; once with the microcolumn method using the DG Gel Coombs card, and once with the conventional tube method. Results between the two methods were compared. The identity of the antibody(s) in each sample was known.
Results: Out of 48 reactions with cells that were positive for the corresponding antigen, the microcolumn method had three false negative reactions, while the tube method had 25. This resulted in the tube method having a lower sensitivity (48%) compared to the microcolumn method (94%). Out of 62 reactions with cells that were negative for the corresponding antigen, there was one false positive reaction in the microcolumn method which lowered the specificity to 98%. The tube method had no false positives and therefore had 100% specificity.
Conclusion: When performing the indirect antiglobulin test, the microcolumn method was found to have significantly higher sensitivity for the detection of antibodies compared to the tube method. This suggests that the microcolumn method is more suitable for routine antibody identification. However, laboratory scientists should follow laboratory protocols and use whichever method is most appropriate at the time.

Evaluation of the performance of the albumin bromocresol purple reagent

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Objectives: The aim of the evaluation was to critically analyse the performance of the Sentinel Diagnostics albumin bromocresol purple reagent on the Beckman Coulter AU680 analyser.
Methods: In this evaluation, a series of tests were performed on the Beckman Coulter AU680 analyser to analyse the precision and accuracy of the method, along with the linearity of the test and stability of the reagent. The reagent was tested using quality controls over 19 days to evaluate the stability. Pooled high and low patient serum was tested both with mixing three times weekly and without, in order to evaluate both the precision and accuracy, and the effect of sedimentation on the reagent. A single bottle of quality control was tested with the routine albumin bromocresol purple reagent to evaluate the stability of the quality control material. Precision was checked at 2 levels using pooled high and low patient serum. Linearity was assessed using a serial dilution of patient samples. Additionally, 2020 external quality control data was assessed.
Results: The albumin bromocresol purple reagent was found to be stable within 2 SD over the 19 day period. The quality control material was stable over the period tested. As found previously, unmixed reagent results drifted. Intra-batch precision was found to be similar to that stated by the manufacturer. The reaction was linear down to about 6 g/L albumin.
Conclusion: The albumin bromocresol purple reagent performed satisfactorily for the requirements of the laboratory. Both the reagent and quality control materials were stable for the manufacturer claimed period of use and the linearity was sufficient for medical purposes.

Verification of the Pathogen 200 v3.1 protocol on the Roche MagNA Pure 24 Diagnostics for the extraction of DNA from faecal samples with comparison to the Pathogen 200 hp protocol

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Objectives: Acute gastrointestinal illness (AGI) creates a significant burden of disease in New Zealand. Pathogenic causes of AGI are explored using Multiplex Tandem (MT)-PCR at Wellington SCL. The quality of nucleic acid extracts has significant effects on the ability of PCR to detect targets within samples. This study aimed to verify an updated extraction protocol (Pathogen 200 v3.1) by comparing its performance against the current extraction protocol (Pathogen 200 hp) used for faecal samples.
Methods: Take-offs were generated using the Faecal Pathogen and Parasite (12-well) assay by AusDiagnostics on nucleic acid extracts from twenty-three faecal aliquots using the current and updated protocols on the Roche MagNA Pure 24 system. Samples were selected to produce a data range that detected each nucleic acid target in the MT-PCR assay at early (≤5 cycles) and late (18-20 cycles) take-offs.
Results: A strong correlation between take-offs produced by extracts from the current and updated protocol was observed ($r = 0.99$, $R^2 = 0.98$). There was no statistically significant difference between protocols ($p = 0.70$). No trend in take-off differences by target was observed.
Conclusion: The updated protocol showed statistically comparable results with the current protocol during MT-PCR analysis to generate take-offs for each pathogen. No change in extraction time was observed. Limitations such as insufficient samples size and extraction failures meant not all targets could be verified for both take-off ranges. Other stated benefits of the updated protocol were not observed. Therefore, there is sufficient but limited evidence to recommend changing the faecal extraction protocol used by WSCL.

Evaluation of DiaSorin aldosterone assay

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Objectives: Aldosterone is a steroid hormone secreted by the adrenal cortex which regulates body fluid volume. Southern Community Laboratories (SCL) Dunedin is utilising a new DiaSorin plasma aldosterone assay on a Liaison analyser to

test patient samples. Currently, all aldosterone tests are sent to Canterbury Health Laboratories (CHL), which uses an IDS-iSYS aldosterone assay. The aim of this research was to evaluate the agreement between DiaSorin aldosterone assay results and IDS-iSYS aldosterone assay results and discover any potential reference interval changes needed.

Methods: The DiaSorin aldosterone assay on the Liaison analyser uses a chemiluminescent competitive immunoassay. In this research, two DiaSorin quality controls (lower 184.2pmol/L; higher 678.7pmol/L) were tested 11 times. Also, 52 patient plasma specimens were collected and analysed by both laboratories with their own aldosterone assays.

Results: Student's t-test (mean difference 45.33pmol/L; 95% confidence interval 17.26-73.39pmol/L; p=0.0021) indicated a statistically significant mean difference between the results obtained at SCL Dunedin compared to CHL. Passing-Bablok fit (y=31.77+0.8696x) showed a negative bias (approximately 13%) for SCL Dunedin results compared to CHL results, which is clinically insignificant when compared to the RCPA Analytical Performance Specification. The SCL Dunedin results were precise according to the coefficient of variation (CV) of QCs (DiaSorin Lower: 10.19%, DiaSorin Higher: 7.43%).

Conclusion: The mean of SCL Dunedin aldosterone assay results was statistically different from the mean of CHL results. However, the SCL Dunedin results had good precision and were clinically comparable to the CHL results with a downwards bias of approximately 13%. Thus, the DiaSorin results and IDS-iSYS results have acceptable agreement and the reference interval does not need to be changed .

Publications by NZIMLS members

This column is to showcase recent international publications by NZIMLS members. Please send details of any recent publications to the Editor at rob.siebers@otago.ac.nz.

Moore JA, Pullon BM, Wang S, Brennan SO. Hb Tacoma: G>T or G>C, and Does It Matter? *Hemoglobin* 2021; 45(3): 203-206.

Abstract

Hb Tacoma [β30(B12)Arg→Ser] is a missense variant that is caused by either an AG*G*>AG*T* or AG*G*>AG*C* substitution at codon 30 of the *HBB* gene. Currently, the latter is classified as a rare cause of β^0-thalassemia (β^0-thal). We propose that *HBB*: c.93G>C has been incorrectly assigned as β^0-thal and discuss whether *HBB*: c.93G>T or *HBB*: c.93G>C should be classified as β^+-thal instead, or as β-globin variants without thalassemic effect. We present several subjects who are heterozygous for Hb Tacoma, one with *HBB*: c.93G>T and two with *HBB*:c.93G>C, to support our conclusions.

Keywords: Hb Tacoma; capillary electrophoresis (CE); splicing; time-of-flight (TOF) mass spectrometry (MS); β0-Thalassemia (β0-thal).

Rivas L, Strydom H', Paine S, Wang J, Wright J**.** Yersiniosis in New Zealand. *Pathogens* 2021; 10(2); 191.

Abstract

The rate of yersiniosis in New Zealand (NZ) is high compared with other developed countries, and rates have been increasing over recent years. Typically, >99% of human cases in NZ are attributed to *Yersinia enterocolitica* (YE), although in 2014, a large outbreak of 220 cases was caused by *Yersinia pseudotuberculosis*. Up until 2012, the most common NZ strain was YE biotype 4. The emergent strain since this time is YE biotype 2/3 serotype O:9. The pathogenic potential of some YE biotypes remains unclear. Most human cases of yersiniosis are considered sporadic without an identifiable source. Key restrictions in previous investigations included insufficient sensitivity for the isolation of *Yersinia* spp. from foods, although foodborne transmission is the most likely route of infection. In NZ, YE has been isolated from a variety of sick and healthy domestic and farm animals but the pathways from zoonotic reservoir to human remain unproven. Whole-genome sequencing provides unprecedented discriminatory power for typing *Yersinia* and is now being applied to NZ epidemiological investigations. A "One-Health" approach is necessary to elucidate the routes of transmission of *Yersinia* and consequently inform targeted interventions for the prevention and management of yersiniosis in NZ.

Aitken JM, Phan K, Bodman SE, Sharma S, Watt A, George PM, Agrawal G, Tie ABM. A Mycobacterium species for Crohn's disease? *Pathology* 2021; S0031-3025(21)00234-8. Online ahead of print.

Abstract

In ruminants Mycobacterium avium subspecies paratuberculosis (MAP) is the causative organism of a chronic granulomatous inflammatory bowel disease called Johne's disease (JD). Some researchers have hypothesised that MAP is also associated with Crohn's disease (CD), an inflammatory bowel disease in humans that shares some histological features of JD. Despite numerous attempts to demonstrate causality by researchers, direct microbiological evidence of MAP involvement in CD remains elusive. Importantly, it has not been possible to reliably and reproducibly demonstrate mycobacteria in the tissue of CD patients. Past attempts to visualise mycobacteria in tissue may have been hampered by the use of stains optimised for Mycobacterium tuberculosis complex (MTB) and the lack of reliable bacteriological culture media for both non-tuberculous mycobacteria (NTM) and cell-wall-deficient mycobacteria (CWDM). Here we describe a Ziehl-Neelsen (ZN) staining method for the demonstration of CWDM in resected tissue from patients with Crohn's disease, revealing the association of CWDM in situ with host tissue reactions, and posit this as a cause of the tissue inflammation. Using the ZN stain described we demonstrated the presence of CWDM in 18 out of 18 excised tissue samples from patients diagnosed as having Crohn's disease, and in zero samples out of 15 non-inflammatory bowel disease controls.

Keywords: CD; CWDM; Crohn's disease; Koch paradox; MAP; Mycobacterium avium subspecies paratuberculosis; NRP; biofilm; cell-wall-deficient mycobacteria; endospores; mycolic acid; non-replicating persistence.

Science Digest

Contributed by Michael Legge

Quality assurance in molecular diagnostics

Since the use of molecular diagnostics over the last 30 years the development and performance has become increasingly complex and with it appropriate quality assurance programmes. Despite the rapid development of new or novel molecular techniques and their application to diagnosis, quality assurance and quality control have not necessarily kept-up with the developments. Examples might be automated PCR, next generation sequencing (NGS), and increasingly the use of molecular diagnostics in Point of Care Testing (POCT). A recently published multi-centre article considers correct quality assurance and looks at some future challenges (1). Considering the status-quo the authors discuss the use of ISO 15189 standards and the use of Standard Operating Procedures; validation, adequacy of training, internal QC, laboratory organization and primer design. External quality assessment is discussed in relation to over 20 proficiency providers and there is a substantial section on issues relating to legal frame works. Looking to the future, the authors again highlight the rapid development of technologies in particular multiplex PCR, Digital PCR and next generation sequencing. They discuss the problems of obtaining appropriate controls and the increasing reliance on bioinformatics. In conclusion, the authors highlight the increasing complexity for quality assurance and the necessity for harmonization and standardization of quality measures.

Be wary of autocorrect

Despite the best designed autocorrect programmes there are still issues with their use. Although most everyday applications, such as extra gaps, various versions of "Spell Check" and punctuation etc, generally work well. However, auto correct can lead to distortions of text or data. In a recent publication from Australia, the authors scanned supplementary files associated with publications from 2014 to 2020. Using scanning software developed by the authors 30.9% (3436/11,117) gene error names were identified when supplementary EXCEL lists were analysed. Although this problem had been previously identified it was not as high as the present authors found. Examples identified were auto-conversion of gene names to dates such as *SEP8* and *MARCH3* which would be incorrectly put in to data bases. They point out that typically these errors may occur in large data-sets. Analysing 166,139 genomics articles they identified gene naming errors by year and organism, and in leading high impact factor journals. They tested spreadsheet software and both Excel and Googlesheets had a high gene nomenclature autocorrections. Two other software programmes, LibreOffice and Gnumeric, were very reliable. The authors recommended that to avoid such errors, scripted analysis is preferred, use LibreOffice as the preferred spreadsheet. If using Excel care and importing data is necessary. Consider using "flat-text files" and verify gene names are intact.

Neonatal sepsis at point of care.

Neonatal sepsis is a life-threatening clinical condition, which may occur at two specific periods: within 72 hours of birth or in neonates that are <28 days old. Typically, early onset is usually due to vertical transmission from the birth canal. However, both routes of infection can result in multiple organ failure and death. A review of neonatal sepsis published this year provides an excellent overview of these infections and developments in their rapid diagnosis. (3). The authors discuss the routine methods for detecting pathogen response and the upregulation of various biomarkers during sepsis. There are well written sections relating to routinely used response biomarkers for sepsis and their limitations. Progressing to the development and the use of sensors to detect neonatal sepsis they present a comprehensive overview of what is available and developments with sensors for the rapid diagnosis of neonatal sepsis. The authors conclude that a combination of biomarkers on a single sensor platform will provide rapid, sensitive, and accurate diagnosis of this infection. The article is well-written and well-illustrated and has a comprehensive reference list, which is up to date.

When best to compete for an Olympic medal?

With the Tokyo Olympic Games now history there will be, no doubt, multiple analysis of athlete's performances and what may have contributed to their successes or failures in performance. Although elite athletes' performances are carefully monitored and appraised little attention has been given to the time of day when the athlete undertakes a competition. An international cooperation between European and USA scientists have analysed Olympic swim times from 2004 to 2016 related to the time of day using publicly available Olympic records and reports (4). Swimming was chosen as it had the least number of variables such as equipment, shoes, climate, etc. In total 144 individual swim times were analyzed including 72 female athletes. Times over heats, semi-finals and finals were analysed per stroke and normalised on an individual basis. The authors then analyzed data in three datasets: race type and time of day, time-of-day only, and magnitude of time-of -day i.e., the difference between first and second placings. The overall outcome of this research was that performance was strongly affected by the time-of-day with the fastest swim times in late afternoon (around 1700hr) with a relative improved performance from morning (0800hr). The time-of-day effects were noted as large and exceeded time differences between gold and silver, silver, and bronze, and bronze and no medals. They conclude that physical performance is not only determined by training but also the athlete's endogenous circadian rhythm system and a possible relationship with core body temperature linked to metabolic homeostasis and circadian rhythms.

Is too much exercise detrimental?

Regular exercise is known to be beneficial for both physical and mental health. But when might be too much? Often high-performance athletes comment that they have "nothing left" after extreme exercise or a competition. A recent Swedish publication may well begin to provide some answers to the issue of "nothing left" (6). Exercise training is known to increase mitochondrial oxidative capacity and improve glucose regulation. The Swedish researchers asked the question, is there an upper limit to the beneficial effects relating to the amount of exercise undertaken? To address this question they had six female and five male healthy volunteers who undertook regular exercise by endurance and strength training on a regular basis. They were all pre-tested on a variety of exercise strategies and their physiological and biochemical responses were analysed. Muscle biopsies were also taken for analysis. Diets were, as far as possible. standardised and samples were taken while fasting. The subjects then undertook progressive set exercise programmes starting with light, moderate, to excessive exercise in a controlled environment over a period of 40 days. This was followed by a monitored recovery period. At the end of each training period they had a GTT and a muscle biopsy as well as other biochemical parameters measured both during and at the end of each training period. Overall the testing period took 40 days. Analysis of the data demonstrated that there was an upper limit for the level of intensive exercise that could be undertaken without disrupting metabolic homeostasis. From the muscle biopsies the limitation of intensive exercise was correlated with a partial shutdown of mitochondrial respiratory function and hydrogen peroxide production. Associated with this was glucose intolerance. The authors strongly recommended that those undertaking strenuous exercise should carefully monitor their body responses to reduce the negative effects of exercise, which could be undertaken by monitoring glucose homeostasis.

Biotin interference
Biotin is available as an over-the-counter supplement, which is widely used by members of the public. Previously it has been demonstrated that doses of >10ng/ml of biotin has the potential to interfere with immunoassays using biotin-streptavidin systems and that approximately 60% of popular immunoassay systems use the biotin linked assays. Many of the publications to date have primarily investigated hormone or related assays and biotin interference. However, a publication from Belgium has investigated biotin interference with the serological markers antiHBs, antiHB core total antibody, and antiHBe. The investigators use healthy volunteers who took a 100mg dose of biotin and a series of pre- and post-biotin blood samples were taken. In addition, anti-HIV/24AG and anti-HCV patient samples were 'spiked' with biotin. Overall, the control (non-infected) pre-biotin administration samples were negative. However, after 1.5 hours post-biotin 80 to 90% of assays showed a significant decrease. The biotin-'spiking' demonstrated a dose dependent concentration effect i.e., as the biotin concentration increased the reliability of both assays decreased. The authors concluded that biotin interference may lead to misdiagnosis with undesirable outcomes and that analysers using the streptavidin-biotin system would be most likely to produce false low results.

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NICE Weekend 2021

The 31st Transfusion Science Special Interest Group (NICE Weekend) was held at Bayview Wairakei over the weekend of 21st – 23rd May this year after a break in 2020 due to COVID restrictions. It was great to meet up face to face again with all our transfusion friends!

54 delegates and trades representatives attended with our resident TMS (Richard Charlewood). Thanks to Roche who supported attendance of our BMLSc student Kyra Chaplin from AUT. Numbers attending were down this year but those attending enjoyed the more intimate feel. There was still plenty of variety in the presentations. Every scientist attending must present either a poster or a 5-minute presentation on anything related to the world of Transfusion. Although it can be nerve-wracking for some, it does provide a great variety of topics from bench level to management and plenty of robust discussion.

The theme was Black & White and the dress-up at Saturday night dinner was again a huge success with some great ideas brought to life! Penguins, Chess boards, The Grim Reaper and more.

Prize-winners

Best Overall Presentation (Sponsored by Abbott):
Colleen Behr – COVID 19 & Stem Cell Collections

Best Poster (Sponsored by TempRecord)
Isabelle Antonio – Vel, Vel, Vel…What do we have here?

Best First-Time presenter - NICEst Virgin (Sponsored by Grifols)
Maxine van Rijn – Chimerism

Most Promising Transfusion Scientist (Sponsored by Ortho Clinical Diagnostics)
Aimee Sanders – Evaluation in Health

Sponsorship to Australia NICE (Sponsored by Immulab – *this year due to COVID restrictions it is to attend NICE NZ 2022*)
Anthony Nalder – A Suspected Anaphylactic Reaction to DMSO

Thanks to all our winners and everyone who presented – you are all awesome and make the judges' task harder every year with the caliber of presentations.

A special thanks to Aous who manages to co-ordinate all the IT requirements whilst still doing his own presentation AND taking notes! 9 TSSIG members attended and helped out in all facets of the weekend, making my job a little less stressful. I salute you all!

See you all next year (hopefully in person!)

Raewyn Cameron – NICE Manager

NICE People 2021

Journal Questionnaire

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 11th February 2022**. You must get a minimum of eight questions right per questionnaire to obtain five CPD points. The Editors set the questions but the CPD Co-Ordinator, Jillian Broadbent, marks the answers.
Direct any queries to her at cpd@nzimls.org.nz.

NOVEMBER 2021 JOURNAL QUESTIONNAIRE

1. The UK guidelines for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) strongly recommends the use of vancomycin for the treatment of MRSA in which serious infections?

2. Which difficulties are encountered with MIC determination?

3. What might the absence of *M. pneumoniae* DNA in nasopharyngeal samples of seropositive CAP individuals be due to?

4. What is the role of fibrinogen in the process of haemostasis?

5. Which factors have been identified as triggers for the onset of sickle cell-related crisis?

6. Elevation of neutrophil concentration in the blood of patients with sickle cell anaemia have been attributed to which mechanisms?

7. What three pillars are used for the diagnosis of celiac disease?

8. The neuronal regulator, Netrin-1, controls which processes?

9. What are the clinical presentation phases of anti-NMDAR encephalitis?

10. The complete blood count in patients with chronic myeloid leukemia typically shows which abnormalities?

ANSWERS AUGUST 2021 QUESTIONNAIRE

1. For community urine antibiotic susceptibility testing which antibiotics should not be reported if pyelonephritis is noted in the clinical details?

   Trimethoprim or nitrofurantoin.

2. Which antibiotics recommended for cystitis should not be reported for blood culture antibiotic testing?

   Cephalexin, trimethoprim, and nitrofurantoin.

3. For blood cultures/sterile sites containing *Pseudomonas aeruginosa* which antibiotics should always be reported for susceptibility testing?

   Ceftazidime, piperacillin-tazobactam and ciprofloxacin.

4. When should susceptibility testing for *Streptococci* groups A, B, C and G be done?

   If from a sterile site, if part of a mixed culture with MRSA, or if allergy to B-lactam antibiotics is stated on the request form.

5. How can selective antimicrobial reporting improve antibiotic use (or benefit antimicrobial stewardship practises)?

   Reduce the use of unnecessary broad spectrum antimicrobials and improve adherence with antimicrobial guidelines for common conditions.

6. In the indoor environment, house dust mites are abundant in which sites?

   Carpets, upholstery, mattresses, and bedding.

7. Which techniques are effective in limiting children's exposure to house dust mites?

   Treating their soft toys by freezing them overnight, hot tumble drying them in a domestic clothes dryer for one hour, or washing them in 0.2% to 0.4% eucalyptus oil.

8. *Naegleria* are what type of amoebae and exist in which forms?

   Unicellular, eukaryotic organisms which exist in amoeboid, flagellate and cyst forms.

9. What are the key CSF features in primary amoebic meningoencephalitis?

   Increased white cell count (300 to >1000/ul) with a prominence of polymorphs, increased protein, decreased glucose, discoloured CSF (white, grey or bloodstained), no organisms seen in gram stain, motile organisms seen while performing cell count.

10. What other species of amoeba, other than *N. fowleri*, can cause CNS infections?

    Acanthamoeba* spp, *Balamuthia mandrillaris, Sapinnia* spp, and *E.histolytica.

Laboratory Christmas quiz: light relief from CPD questions.

This year the NZIMLS Council has agreed that there should be a bit of light relief towards the end of 2021. A general knowledge quiz has been created by an anonymous Quiz Master and the NZIMLS Council has agreed that a single prize of **$200** towards a laboratory Christmas function will be awarded to the most correct answers. While individuals can also complete the quiz, the prize will go only to a laboratory entry to encourage teamwork. There are **no** CPD points for this quiz, and it is not discipline related. The NZIMLS Council has not participated in the question setting or have knowledge of the answers. An answer sheet is available on the NZIMLS website and the quiz answers can be submitted electronically (as for the CPD Journal questions). The answers will be published on the NZIMLS website after the quiz closes. Closing date for all answers is December 1st and no late entries will be accepted.

1. Which of the following would describe gremolata?
   a. Spanish condiment
   b. Italian condiment
   c. French condiment
   d. Portuguese condiment

2. Which of the following car logos uses the constellation Matariki?
   a. Toyota
   b. Saab
   c. Subaru
   d. Lotus

3. Which of the following is not a duck?
   a. Bombay
   b. Peking
   c. Mallard
   d. Blue

4. Which of the following is most energy dense?
   a. Carbohydrate
   b. Protein
   c. Alcohol
   d. Fat

5. Which of the following breads does not require yeast?
   a. Pretzels
   b. Poori
   c. Pitta
   d. Hamantasche

6. Which of the following communication systems was set up by scientists working on an atomic physics project?
   a. Fax
   b. Internet
   c. Computer operating systems
   d. Microwave data transmission

7. Which of the following authors had 30 first novel rejections before it became a best seller?
   a. Stephen King
   b. JK Rowling
   c. George Orwell
   d. John Le Carre

8. New Zealand jade comes from which type of rock?
   a. Nephrite
   b. Granite
   c. Garnet
   d. Pounamu

9. Which fruit is the world's largest edible herb?
   a. Avocado
   b. Mango
   c. Pineapple
   d. Banana

10. The word organic means?
    a. No additives
    b. Nitrogen-based
    c. Naturally occurring
    d. Carbon-based

11. Who was the first person to perfect and install the flushing toilet in Victorian England?
    a. Porcelain Chambers
    b. Josiah Wedgwood
    c. Thomas Crapper
    d. Henry Doulton

12. Of the following, who objected to the use of smallpox vaccinations?
    a. Joseph Lister
    b. Florence Nightingale
    c. Edward Jenner
    d. Louis Pasteur

13. Which ancient civilization developed the use of the right-angled triangle defining the 3,4.5 'rule'?
    a. Egyptians
    b. Babylonians
    c. Romans
    d. Greeks

14. Which culture first identified that using cow pox could protect against small pox?
    a. English
    b. Turks
    c. Chinese
    d. Indians

15. Which ancient Greek philosopher incorrectly had the most significant impact on Western medicine for over 2000 years?
    a. Archimedes
    b. Homer
    c. Hippocrates
    d. Aristotle

16. Which ancient civilization invented the lunar calendar that became the basis of Jewish and Christian religious calendars?
    a. Sumerians
    b. Greeks
    c. Indus Valley
    d. Egyptians

17. The larger-phone is which of the following?
    a. A member of the early 18th century bassoon instruments
    b. A Carlsberg customer service number
    c. A rhythm instrument in a bush band
    d. A medieval wind instrument

18. Which of the following New Zealand Prime Ministers died while in office?
    a. Norman Kirk
    b. Keith Holyoake
    c. Peter Fraser
    d. Walter Nash

19. Which Antarctic explorer stored Scotch whiskey under the floor boards of the hut?
    a. Mawson
    b. Shackleton
    c. Scott
    d. Amundsen

20. "Honey barrels" in the Scotch whiskey industry are due to which of the following?
    a. Charring inside the barrel
    b. Use of alternative differing oak staves
    c. Being placed near a window
    d. Being placed near bee hives

21. Which of the following group of pharmaceuticals had their discovery based on Mexican yams?
    a. Chemotherapy agents
    b. Antidepressants
    c. Antimalarials
    d. Oral contraceptives

22. Which physicist finally worked out the nuclear chemistry of the sun in an American dining car on serviettes?
    a. Niels Bohr
    b. Max Plank
    c. Fred Hoyle
    d. Albert Einstein

23. Which of the following female scientists made a discovery that provided the basis for the DNA double helix discovery?
    a. Dorothy Hodgkin
    b. Rosalind Franklin
    c. Mary Sommerville
    d. Rosalyn Sussman

24. Who invented the petri dish and named it after one of laboratory assistants?
    a. Robert Koch
    b. Louis Pasteur
    c. Alexandra Fleming
    d. Joseph Lister

25. Which of the following countries has banned Christmas day?
    a. Vietnam
    b. Indonesia
    c. Cuba
    d. Brunei

26. Which of the following countries typically celebrates Christmas day dinner with KFC?
    a. Slovakia
    b. Canada
    c. Japan
    d. Phillippines

27. From the influenza pandemic in Europe in 1510 the word influenza was first used. From which country did this word originate?
    a. Spain
    b. Italy
    c. France
    d. England

28. In which country was the modern helicopter developed?
    a. England
    b. USA
    c. Germany
    d. Italy

29. The disease described as "French disease" was historically used to describe which disease?
    a. Syphilis
    b. The plague
    c. Gonorrhea
    d. Smallpox

30. Which book was Arthur Conan Doyle's first Sherlock Holmes book?
    a. The Sign of Four
    b. Hound of the Baskervilles
    c. A Study in Scarlet
    d. The Valley of Fear

31. What was the substance that Sherlock Holmes considered important in solving crimes?
    a. Haemoglobin
    b. Hair
    c. Saliva
    d. Skin

32. Which of the following composer's music determined the recording length of the original compact discs?
    a. Handel
    b. Mozart
    c. Beethoven
    d. Tchaikovsky

33. Which country invented the horse stirrup?
    a. Japan
    b. England
    c. China
    d. Italy

34. Which religious faith created the first medical teaching hospitals?
    a. Christians
    b. Judaism
    c. Buddhism
    d. Islam

35. Which was the first town in New Zealand to get electricity generated for public use?
    a. Greymouth
    b. Reefton
    c. Gore
    d. Darfield

36. In the eighteenth century, which country was the leading country for the use of algebra in science?
    a. France
    b. Britain
    c. Germany
    d. Holland

37. In which country did the use of "Ordinance Survey' now used with maps originate?
    a. England
    b. Scotland
    c. Ireland
    d. Wales

38. From which branch of science did the Gaussian curve used in statistics develop from?
    a. Biology
    b. Physics
    c. Geology
    d. Astronomy

39. Koch's postulates are based on which of the following?
    a. The identity of tuberculosis
    b. A clear set of experimental procedures
    c. That bacteria can cause disease
    d. That better social conditions reduce the incidence of disease

40. Which country initiated the use of decimal time e.g. 10-hour clocks, 10 months per year?
    a. Russia
    b. Germany
    c. France
    d. Spain

41. From which culture did the word alcohol originate?
    a. Turkish
    b. Arabic
    c. Armenian
    d. Egyptian

42. What pressure requirement is required for a champagne bottle?
    a. One atmosphere
    b. Four atmospheres
    c. Six atmospheres
    d. Ten atmospheres

43. Historically, which of the following was accidentally named after a Viking name for a stream in Yorkshire, England?
    a. Folgers
    b. Starbucks
    c. Moccona
    d. Costa

44. Cappuccino coffee was named after which of the following?
    a. Cappuccino hooded monkeys
    b. Cappuccino Italian fashion line
    c. Cappuccino rabbits
    d. Cappuccino monks clothing

45. The Receiver Operating Curve (ROC Curve) was originally created for what of the following purposes?
    a. Discrimination of radio waves
    c. Determining statistical probability
    d. Determining areas under the curves
    e. Submarine detection with sonar

46. Which is the only letter not appearing in the periodic table of elements?
    a. Z
    b. X
    c. J
    d. W

47. What is the maximum number of times that a piece of paper can be folded?
    a. 7
    b. 10
    c. 8
    d. 11

48. The communication term "Bluetooth" was named after which of the following?
    a. King Harold 1 of Denmark
    b. King Egbert of England
    c. King Harold of Norway
    d. King Athelred of England

49. Castor oil (an early well-known laxative) was originally obtained from which of the following?
    a. Ambergris from sperm whales
    b. Beaver groin glands
    c. Rendered penguin oil
    d. Seal oil

50. A Margherita pizza is named in honour of which of the following?
    a. Italian drink
    b. Italian pizza maker's wife
    c. Italian town
    d. Italian queen

Load your answers online at

https://www.nzimls.org.nz/journal-christmas-quiz.html

Executive Office Christmas Hours

The NZIMLS Executive Office will be closed for the Christmas break from Thursday 23 December 2021, re-opening on Monday 10 January 2022

We would like to wish you all a very safe and happy holiday period and thank you for your dedication and hard work throughout 2021

Meri Kirihimete me te Tau Hou hari

Merry Christmas and Happy New Year

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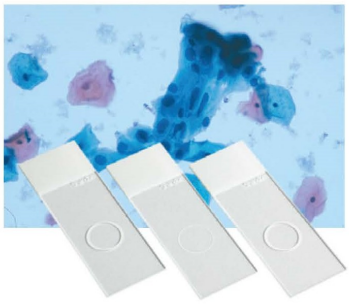

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