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In this issue

Rob Siebers, Editor

Arleen Donaldson and Mackenzie Nicol from Aotea Pathology, Wellington investigated whether enrichment of clinical faecal specimens in Selenite F broth, for the cultivation of *Salmonella* and *Shigella,* should be discontinued in the community laboratory setting. They showed that enrichment in Selenite F broth should continue to be used and a combination of direct plating and sub-culturing is required in order to maximise the recovery rate of both *Salmonella* and *Shigella* as well as to provide early detection. If enrichment was to be discontinued, potentially a significant proportion of *Salmonella* and *Shigella* isolates may have been missed (61% and 33% respectively.

Studies have reported that *Helicobacter pylori* infection may be implicated in chronic immune thrombocytopenia and in some cases infection eradication has returned the platelet count within the reference range. Noah Sibanda and colleagues investigated whether the platelet counts of people with *H. pylori* infection were lower than those without the infection. In addition they sought to establish whether infection with cagA positive strains affected the platelet count more than cagA negative strain infections. Furthermore, they also investigated whether eradication of *H. pylori* led to an increase in the platelet count. Results from their study showed some difference between the mean platelet counts of *H. pylori* infected and uninfected patients. Patients infected with cagA positive strains, tended to have a lower mean platelet count compared to those with cagA negative strains. Eradication of *H. pylori* infection failed to make a significant difference to the platelet count.

Resistance to carbapenems, particularly due to acquired carbapenemases, in Gram-negative bacilli continues to disseminate worldwide. A range of chromogenic screening media have been developed for the selective differentiation of carbapenem-resistant Gram-negative bacilli. CHROMagar™mSuperCARBA™ has recently become available in New Zealand and was evaluated against the current screening protocol for the recovery of carbapenemase producing organisms in faecal screening samples by Julie Creighton and Hui Wang from Canterbury Health Laboratories, Christchurch. They found that the current screening method consisting of CHROMagar™ ESBL (CESB) and MacConkey agar with a 10 µg meropenem disc was found to have the best overall performance. CHROMagar™mSuperCARBA™ showed excellent sensitivity and specificity for the recovery of CPO, and would be considered a beneficial addition in an outbreak situation.

Katie Anderson and James Faed from Dunedin investigated whether visible aggregate material was present in stored New Zealand red cell units and whether these could potentially be trapped in the 170-200 micron administration set filters after the passage of one unit of re-suspended red cells. They found negligible visible aggregated material in the protein assays of flushing solutions. Mass spectrometry identified that the major protein was haemoglobin. They concluded that that current storage procedures and processing to remove leucocytes and platelets, satisfactorily prevents formation of aggregated particulates of leucocytes, platelets and fibrinogen.

Both Noonan syndrome and juvenile myelomonocytic leukemia are characterised by hyperactivation of the Ras/MAPK signalling pathway, and as such may manifest concurrently. Ceryn Hutin and colleagues from Rotorua and Auckland report a case study involving twins who originally presented with a petechial rash and a marked thrombocytopenia. Over time, the haematological picture developed into a juvenile myelomonocytic leukemia, and given the rare nature of this disorder, prompted review for other clinical manifestations, thus ultimately allowing the haematologist to consider a syndromic disorder.

In this issue, Michael Legge from Otago University presents a retrospective look at the some important biochemical methods available in 1957 in the clinical laboratory, and their interpretation is presented with the principles of the methods. He concludes that over the last 60 years there have been dramatic developments in clinical biochemistry. As clinical demands evolved new approaches to diagnostic testing were required such as the development of flame photometry for sodium and potassium and the concept of diagnostic test profiles.

Each year, Council of the NZIMLS invites a scientist or pathologist, who has made a significant contribution to laboratory medicine in New Zealand, to deliver the prestigious TH Pullar Memorial Address at its Annual Scientific Meeting. This year's address was given by Russell Cole from the Pacific Paramedical Training Centre (PPTC) in Wellington. Russell's topic was the empowering impact of quality systems and the challenges of trying to impose the high New Zealand standards on resource-poor countries in the South Pacific region .

TH PULLAR MEMORIAL ADDRESS

The empowering impact of quality systems

Russell Cole

Pacific Paramedical Training Centre, Wellington

Tena Koutu, Talofa Lava, Kia Orana, Malo e Lelei, Bula Vinaka, and Good Morning to you all. It is an amazing privilege to be standing here giving this address, I am both honoured, humbled, and quietly amused all at the same time as I know many finer, smarter, more astute scientists have been here before me. My only boastful claim would be that I wonder how many could compare with my intrepid travels, somewhat flamboyant journey, and enjoyable vibrant career experiences.

A career in medical laboratory science has seen me on the shores of the Solomon Islands, sitting in the front row of a World Health Organisation meeting in Manila, being escorted through the military base of Kwajaleen in the Marshall Islands, talking laboratory to Secretaries of Health, training the locals of Nauru, Tuvalu, Ebeye, and Santo, even eating taro for lunch at Savaii Hospital. Impacting experiences and wonderful people. However, back into reality, a big thank you to my Bay of Plenty colleagues for dobbing me in and the gift of sleepless nights and stressful anticipation that comes with this occasion. Regardless of that, it's fantastic to be back in the Bay where everything grows abundantly, the geysers are boiling, the surf is always pumping, and the mud smells like it just came out of the autoclave.

Undoubtedly one of the nicest regions of New Zealand but as we know the true gold isn't found in the hills of Waihi, but in the people. Good down to earth Kiwi's giving all for their local communities, proud and passionate in a land of plenty. After being born and bred on the slopes of Mt Albert and graduating through the now extinct Auckland Hospital School of Laboratory Technology, I spent a number of wonderful years experiencing the finer points of laboratory management at Whakatane Hospital. A close-knit community based hospital, it became known as the country's smallest CHE, and aptly named Eastern BOP CHE (Crown Health Enterprise) under the Labour government of the early 90's.

In amongst those traumatic times of sweeping health reforms and frozen budgets, the laboratory rode the tide of aggressive industrial relation matters, staff retention and recruitment problems, community market competition on the hospital front door and the imposing influence of a rising organisation called TELARC. (IANZ from 1997). Strangely enough during those tumultuous times, the medical laboratory's greatest emerging concern and largest impacting factor was the threatening need to implement and comply to accreditation standards. The emerging pressure on all Laboratories at the time was the directive to achieve and comply to international quality standards of practice throughout all the service components expected in a diagnostic laboratory.

This somewhat daunting challenge was thrown at the feet of a bunch of multi-skilled talented scientists in a medium sized provincial laboratory in the Bay of Plenty. Staff were well trained, committed and high on experience, but unfamiliar or unacquainted with the encroaching quality management systems wave sweeping through the country. Nor did we have any idea of the foreseeable impact such an approach would have on our daily workflow patterns and our traditional well established practices. That year it was presented as a do or die ultimatum from Government to be addressed and achieved in all laboratories whether they were CHE-based or private. For us, it was an ultimatum within 10 months. Suddenly it had become very serious. We had left it that late. The realisation kicked in that this thing wasn't going away. Our laboratory's survival became critical. It depended on achieving accreditation standards or face the consequences of failing to deliver and not being fit for service. Funding contracts would only go to accredited laboratories.

Like many of you with similar experiences our road to redemption was in applying quality principles and documentation standards to every function and process occurring in the laboratory. Every imaginable situation known to man needed solid documentation policies, defining, standardising, and implementing to fit the requirements. Paperwork was developed for newly introduced concepts like a staff orientation programme, competency records, specimen reception SOP's, identifying key performance indicators. Although QC was always an integral part of daily work practices in the 90's, now it had evolved and integrated into a far larger concept - that of quality systems. A bigger picture emerged involving the organogram of the whole organisation and 12 quality system essentials. What made it special too, was the pioneering laboratory driven model that paved the way for the wider hospital clinical departments to embrace a new quality initiative also making a rapid rise – that of healthcare management standards. How fitting for the laboratory to lead the way.

Well the rest is history, amazingly 20 years at least, but more to the point, champions were made that year, individuals knitted into a team like never before, a common pressure and commitment drew us together. Undoubtedly "*team work makes the dream work*", to quote John Maxwell, and yes, gold was created in a remote provincial hospital laboratory in the Eastern Bay. Miles away from the Olympics, yet we made the top of the podium in our turbulent scientific world. Many fine laboratories throughout the country duplicated that scenario, in fact most of them well before Whakatane. However, for us the impact of implementing quality standards was undeniable. An average laboratory was transformed into a finely tuned unit, every dollar and action accounted for, every blood test scrutinized, every specimen request monitored, and followed through to completion.

A lot of water has gone under the bridge since that time and every laboratory in the country has honed and refined its professionalism in quality management systems. Today you can sense the culture and personality of an organisation by what is expressed and stated in its quality manual. Almost like "*smelling the uranium on his breath*" as David Lange once said, you can smell quality systems in the atmosphere. It's the way a laboratory reveals its personality. It shows in patient service policies. The wording used in handbooks and information brochures. The presentation vibes expressed in result reports, request forms and in the screeds of service documentation. The impact of quality standards is frighteningly obvious.

In today's laboratories there are SOP's for Africa, audits for every section, competency training logs developed to the nth degree, hazard and risk assessments ranking every potential harm, and management review meetings to rival any major corporate AGM. Electronic filing systems have taken over because we've got too many policies to manage, human errors are tracked by data collection systems, performance indicators for staff are accumulated daily, and workflow algorithms operate so we don't have to think too much. Clerical audits demanding 100%. Measurable goals and outcomes for every facet of lab work exist. Hopefully you're not too scared to come to work tomorrow because we've got your performance appraisal, remember!

In the name of quality improvement, our unquenchable desire to pursuit excellence and perfection has made quality management into a new science. Arguably it's in our blood, isn't this what we've been trained for, military precision executed into every service function of a laboratory. It's the scientific way, part of our DNA to be seen to be doing the right thing. A never-ending finish line beckons in our obsessive pursuit of excellence. Modern catch phrases fill today's environment like validation, approval, verified, downtime, traceability, allowable limits of performance, even auto authorising accompanies every result released to the outside world. We pride ourselves on our ability to continuously improve everything, standardise everyone's techniques, eliminating all inefficiencies and wastefulness. All part of our service approach of course.

In the quiet, one is left to ponder, Do you think we may have taken things to the extreme ? Is there a possibility of being over the top with quality requirements! Has the pendulum swung too far! Wow! Is this anything like what we envisaged all those years ago. Probably not, we were too busy celebrating that we'd made the grade. It was quality standards that won us the respect and trust of other health services and hospital departments. It was quality standards achievements that elevated us onto the same playing field of international establishments. In a strange way it was quality standards that created a common bond between private and hospital laboratories, even an air of competitiveness crept in because in some cases we had different operating systems and practices upholding the same accreditation status. How was that going to work?

On the down side, what has happened to all the fun we used to have? Is it still an enjoyable and rewarding profession or have we squeezed out, standardised and controlled fun times as though they were work hazards. I'm sure we all have laughable memories of April Fools jokes and high-jinx actions during our training years. Imagine how that would be handled in today's environment – splattered over the pages of an incident report, investigated within 24 hours – because that's a listed key performance indicator from the quality plan, a black mark would find itself on your performance appraisal, you may not be shot at dawn but probably doing time in the level 3 containment facility. Who knows you might even make the list of non-compliance incidents at the annual management review meeting. Either way, we take quality systems and our profession very seriously today. Long gone are the days of playing guitars on night shift, socialising in the wards or sending samples of cake via the orderlies to working colleagues. We're far more accountable in today's environment.

In recent years I've had the privilege of being part of NZ's health development work in the Pacific. You can be very proud of NZ Aid's work across the Pacific basin, making significant impact and valuable improvements in development, not necessarily in healthcare, in projecting $1Billion in aid over the next 3 years (*NZ Aid Strategic Plan 2015-19). Being part of the dynamic team at the Wellington-based Pacific Paramedical Training Centre (PPTC) has brought many amusing but satisfying challenges in the colourful Pacific Island environment of coconut palms, sinking coral atolls and non-accredited laboratories. As a dear Marshall Island pathologist put it "*it's like you guys have a missionary zeal across the Pacific preaching the good news of quality standards*". Oh yes, quality standards exist - but not all of them, systems are progressing in development but there are still significant gaps, performance measures are recognised but not made accountable to. Vision and momentum to achieve excellence is all a bit hazy. Flashbacks to the NZ lab scene in the early 90's is a common occurrence and I find myself often questioning our somewhat arrogant right to impose a quality culture on an often under-resourced, under-appreciated and many times fragmented healthcare service. It has presented a fantastic opportunity to make a significant contribution into the development of medical laboratories. An opportunity to implement our New Zealand laboratory model into Pacifika. The satisfaction comes in helping them grasp the vision of what an accredited laboratory looks like, to be instrumental in inspiring and mentoring their progress, watching them build structure and orderly systems into their work. Many of the same frustrations and questions we pondered over back in the early 90's have been resurrected again across the benches of Pacific Island laboratories, only this time it goes a bit deeper.

Is it fair to drive quality standards, compliance and best practice in to smaller nations suffering with so many gaps in their basic healthcare? Is it reasonable to inflict our Western culture's obsession for achievement, accountability, consistency and accuracy into the Pacific Islander's work ethic? Or again, to impose NZ big brother systems and tactics in an effort to control the fun-loving, jovial culture of Polynesia, Melanesia and Micronesia. On reflection, wisdom tells us that building a house in Pacifika, is not like building a house in NZ. We have to realise that it may have familiar structures and form but assembled with different materials, different support beams holding it in place, different design features customised for a different culture. We shouldn't expect a NZ style home in a Pacific Island setting, arrogant in the knowledge that we've created great labs, excellent systems and recognised professionalism in our home environment. NZ laboratories are leading the world in the way we implement and apply quality standard concepts across the country, but wisdom comes in the way we impart or project that intellectual knowledge in a working partnership with our Pacific neighbours. Pacific Island laboratories need to be allowed to build their own houses, taking ownership of their own destiny and being fully engaged in achieving every aspect of the quality journey. Let them discover their own story as we mentor their development. And as the powerful theme of this year's conference suggests, it's in working together, not in domination or in wishing to impose advice, that is the essence of a successful laboratory service.

Thanks for listening, Fa'afetai, Ka Kite Anu, and I wish you all a fantastic conference.

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ORIGINAL ARTICLE

Evaluation of Selenite F broth as an enrichment step for the isolation of *Salmonella* and *Shigella* in clinical faecal specimens –A retrospective study

Arleen Donaldson and Mackenzie Nicol
Aotea Pathology, Wellington

ABSTRACT

Objectives: To investigate whether enrichment of clinical faecal specimens in Selenite F broth, for the cultivation of *Salmonella* and *Shigella,* should be discontinued in the community laboratory setting.
Methods: A retrospective analysis of all faecal specimens submitted to Aotea Pathology Limited for bacterial culture over a two-year period was analysed using the Labsolutions statistics software. Samples positive for either *Salmonella* or *Shigella* were analysed as being isolated from either the direct Hektoen agar plate, isolated on secondary XLD agar plate following enrichment in Selenite F broth, or both.
Results: Direct culture on Hektoen agar yielded a recovery rate of 39% of the *Salmonella* isolated and 67% of the *Shigella* isolated. Secondary sub culture on XLD agar following enrichment in Selenite F broth yielded a recovery rate of 99% of the *Salmonella* isolated and 50% of the *Shigella* isolated.
Conclusions: This study shows that enrichment in Selenite F broth should continue to be used and a combination of direct plating and sub-culturing is required in order to maximise the recovery rate of both *Salmonella* and *Shigella* as well as to provide early detection. If enrichment was to be discontinued, potentially a significant proportion of *Salmonella* and *Shigella* isolates may have been missed (61% and 33% respectively).
Key words: Selenite F broth, Salmonella, Shigella.

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INTRODUCTION

Salmonella and *Shigella* are members of the *Enterobacteriaceae* family and are non-lactose fermenting (NLF) Gram-negative bacilli causing gastrointestinal illness in humans and are primarily acquired through ingestion of contaminated food and water (1). *Salmonella* can cause enteritis or enteric fever and the severity of disease depends on the inoculating dose, serotype and predisposing host factors (1,2). *Shigella* causes dysentery where symptoms include fever, abdominal cramps, bloody diarrhoea and haemolytic uremic syndrome, the most serious complication of shigellosis (2).

Diagnosis in medical laboratories is mainly by faecal culture, however, molecular testing methods have become available in recent years (2). At Aotea Pathology the protocol for the cultivation and isolation of *Salmonella* and *Shigella* from clinical faecal specimens involved directly plating faeces onto Hektoen enteric agar (half plate) and sub-culturing onto Xylose Lysine Deoxychocolate agar (XLD) following enrichment in Selenite F broth after overnight incubation (All media supplied by Fort Richard Laboratories Limited). Historically, enrichment broths have been a critical step in enhancing the growth of some pathogens while inhibiting other commensal bacteria and are commonly used in combination with direct plating in medical laboratories to ensure recovery of faecal pathogens from diarrhoeal patients (3). It has been suggested that enrichment broths be used only during outbreaks, or for screening asymptomatic carriage, and that it is not a requirement in hospital laboratories as it's primary role is to diagnose acute phase of disease (4,5). Aotea Pathology is also required to diagnose acute diarrhoea. The use of enrichment broths has been discontinued in some laboratories as the yield does not justify the cost and it has been suggested that individual laboratories should look through historic data to help determine whether enrichment broths should remain to be used or discontinued (2).

The aim of this study was to compare direct culture isolates on Hektoen agar to subculture isolates on XLD agar following enrichment in Selenite F broth and to evaluate whether enrichment in Selenite F broth should be discontinued. This was achieved by doing a retrospective study on all positive *Salmonella* and *Shigella* isolated from these media at Aotea Pathology over a two year period.

METHODS

A retrospective study comparing direct faecal culture isolates on Hektoen agar vs sub-cultured isolates on XLD agar after enrichment in Selenite F broth for the isolation of *Salmonella* and *Shigella* from clinical faecal specimens received at Aotea Pathology over a two year period (1 January 2013- 31 December 2014) was performed using the Statistics software linked to the Laboratory Information System, Labsolutions.

Positive samples for *Salmonella* or *Shigella* within the chosen timeframe were searched and analysed as to whether the isolate had been isolated from either direct culture, on subculture following the enrichment step, or both. A total of 190,664 faecal specimens were requested for bacterial culture in the two year period and of these, 172 were positive for either *Salmonella* or *Shigella*. Positive isolates from External Quality Assurance samples were also included in this study.

All media was incubated 18-24 hours at 35^{0}C in O_2, prior to being sub-cultured or read. NLF colonies on either Hektoen or XLD underwent an intermediate step involving a purity plate onto MacConkey agar and subbed onto a urea slope and incubated 18-24 hours at 35^{0}C in O_2. Isolates that were urease negative were then identified by API10S and or API20E (Biomerieux) and serology testing with antisera *Salmonella* polyvalent O (A-S), *Salmonella* polyvalent H (phase 1 and 2), and *Shigella sonnei* (phase 1-2) (manufactured by Remel) was performed on isolates that had included *Salmonella spp* or *Shigella spp* in the API profile. Further characterisation and speciation of all positive isolates was performed by Environmental Science and Research Limited laboratory (ESR) at Wallaceville. All media used was manufactured and obtained from Fort Richard Laboratories Limited where they obtain their reagents to make up media from Becton Dickinson

RESULTS

There were a total of 154 *Salmonella* isolates and of these, 2 were recovered from direct culture only on Hektoen agar compared to 94 on XLD agar only (following enrichment in selenite F broth) and 58 were isolated on both direct and subculture plates (Table 1). There were a total of 18 *Shigella* isolates and of these, 9 were recovered from direct culture only on Hektoen agar compared to 6 from subculture onto XLD agar only (following enrichment in selenite F broth) and 3 were isolated on both direct and subculture plates. When comparing direct culture with subculture only, 60 *Salmonella* isolates were recovered on Hektoen compared to 152 on XLD (following enrichment) and 12 *Shigella* isolates were recovered on Hektoen compared to 9 on XLD (following enrichment).

Table 1. Isolates recovered on Hektoen Agar in comparison to XLD agar following enrichment in Selenite F broth.

		Direct Hektoen agar only	Subcultured XLD only (following enrichment)	Both direct and subculture plates	Total on Hektoen	Total on XLD (following enrichment)
Salmonella (Total of 154)	Isolated	2	94	58	60	152
	Not isolated	152	60	96	94	2
Shigella isolates (Total of 18)	Isolated	9	6	3	12	9
	Not isolated	9	12	15	6	9

The recovery rate on Hektoen agar was 39% for *Salmonella* and 67% for *Shigella* whereas the recovery rate on XLD agar yielded 99% for *Salmonella* and 50% for *Shigella*. There is a slightly higher recovery rate of *Shigella* on Hektoen agar in comparison to XLD but a third of all *Shigella* isolates recovered were not isolated on Hektoen (Table 2).

Table 2. Percentage recovery rate of the total *Salmonella* and *Shigella* isolates recovered on Hektoen agar XLD agar after enrich-ment in Selenite F broth.

	Recovery rate obtained on direct culture onto Hektoen (%)	Recovery rate obtained on Subculture onto XLD (following enrichment in Selenite F broth) (%)
Salmonella spp	39	99
Shigella spp	67	50

DISCUSSION

The practice of using enrichment broths as part of routine culture has been discontinued in some laboratories and it has been suggested that analysis of historic data should be performed to determine whether enrichment broths should remain in use or be discontinued (2). A previous study evaluating Gram-negative enrichment in faecal cultures showed that the use of Gram-negative enrichment broth was at least as good as direct plating as theoretically, direct plating on both Hektoen and XLD would allow selective isolation of *Salmonella* and *Shigella*, making the use of Gram-negative broth unnecessary (4,5).

The aim of this study was to evaluate the possibility of discontinuing the use of Selenite F broth at Aotea Pathology by looking through historic data and comparing direct culture isolates to isolates isolated following subculture from enrichment broth. The results show that enrichment in Selenite F broth potentially maximises the recovery of *Salmonella* in faecal specimens. The recovery rate on Hektoen agar yielded 39% of *Salmonella* isolated whereas on XLD agar following enrichment, the yield of *Salmonella* recovered had increased to 99%. Therefore, if enrichment was to be discontinued, potentially 61% of *Salmonella* isolates may have been missed, which is a significant proportion. Direct culture onto Hektoen agar did have a slightly higher recovery rate of *Shigella* , 67% in comparison to 50% isolated on XLD agar following enrichment. However, surprisingly a third of all isolates were still not isolated on Hektoen and this suggests that both primary and sub-culturing following enrichment is still required in order to maximise the recovery rate of *Shigella*. Direct plating onto Hektoen, in combination with subculture following enrichment, should also continue to be used for the isolation of *Salmonella.* Of the 154 *Salmonella* isolated, 58 were isolated on both direct culture and subculture resulting in earlier identification and therefore reporting of the result. Using a combination of both methods allows early identification of pathogens that is important for public health interventions and to minimise further transmission, especially during outbreaks (7). This result is similar in outcome to a study done in the evaluation of primary inoculation on XLD and following enrichment in Selenite broth. The results from that study showed that direct plating enhances the speed, but not the sensitivity of *Salmonella enterica* (7). Another study evaluating a variety of chromogenic agars and Hektoen agar also showed the importance of using selenite broth for the recovery of *Salmonella* following prolonged incubation of 48 hours, and that direct plating identified *Salmonella* a day earlier in 50% of cases (8).

One of the major limitations of our study was the difference in media used between direct and subculture plates.

As a result, specificity and sensitivity of Selenite F broth could not be calculated and would not have been an appropriate measure in comparison as it would have been difficult to differentiate as to whether the increased performance in the recovery of *Salmonella* and *Shigella* on subculture was due to XLD or Selenite F broth. A study evaluating the use of a variety of enrichment broths and plating media, including Hektoen and XLD for the isolation of *Salmonella spp,* had shown no statistical significance in *Salmonella* positive stools between Hektoen and XLD from direct plating (9). In an external performance evaluation performed by BD in comparing a chromagenic plating medium to XLD and Hektoen, the sensitivity and specificity of Hektoen and XLD were similar (10). Fort Richard Laboratories obtain their media ingredients from BD and therefore if it was assumed that both agars are similar in performance, then these results would indicate that enrichment in Selenite F broth is beneficial for the maximum recovery of *Salmonella* in clinical specimens, and therefore should not be discontinued in the laboratory.

As many members of the *Enterobacteriaceae* are NLF, it is possible to have enteric flora overgrown or mixed in populations with either *Salmonella* or *Shigella* on Hektoen agar, especially when they are present in low numbers. Therefore, there may have been a few colonies on Hektoen that were mixed in with other NLF colonies that may have potentially been missed as a similar colonial morphology was picked instead. Selenite F broth was developed to maximise the chance of isolating and recovering *Salmonella* as selenite is inhibitory for enteric flora and thus enrichment can result in isolation without the overwhelming growth of many enteric flora as long as the incubation period does not exceed 24 hours (6,11). Therefore, due to the inhibitory effects on enteric flora, Selenite F broth is essential to ensure a greater chance of recovering both pathogens. According to BD, the use of Selenite F broth on its own for subculture is not recommended and should be used in conjunction with other selective media to increase the probability of isolating pathogens when present in low numbers (6).

CONCLUSION

Our results showed that direct plating onto Hektoen yields a lower recovery rate in *Salmonella* and the use of Selenite F broth results in an increased yield of *Salmonella* isolated. If enrichment is to be discontinued, potentially a significant proportion of *Salmonella* isolates (61%) may have been missed. However, in order to achieve greater isolation rates, a combination of both direct plating and subculture following enrichment should be performed in order to maximise the recovery rate. Our results also show that a combination of both methods is also required in order to maximise the recovery of *Shigella.* If enrichment is to be discontinued, a third of *Shigella* isolates may have been missed. Direct plating and sub-culturing following enrichment can provide earlier detection and isolation of either pathogen in addition to increased recovery rates. However, due to limitations in our study, ideally a further study where both media were tested in parallel should be performed where the sensitivity and specificity can be calculated in order to truly evaluate the effectiveness of Selenite F broth, and whether there are potential variations in agar performance between Hektoen and XLD.

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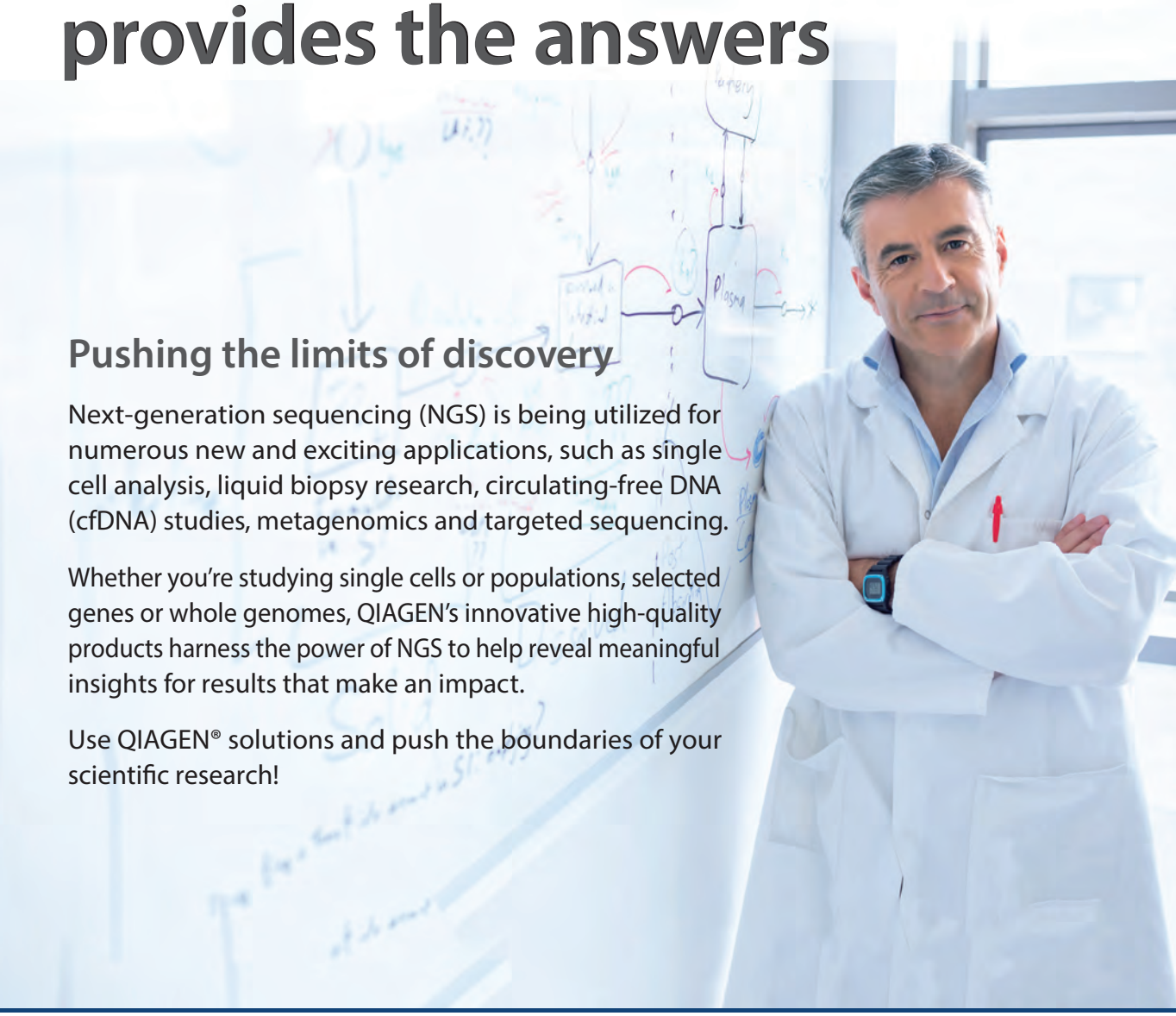

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ORIGINAL ARTICLE

Helicobacter pylori infection and the platelet count

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ABSTRACT

Objectives: Helicobacter pylori (*H. pylori)* infection is a risk factor for the development of gastric and duodenal ulcers and may also lead to cancer. Studies have reported that *H. pylori* infection may also be implicated in chronic immune thrombocytopenia (cITP) which is characterised by the immune-mediated destruction of circulating platelets. In Japan, *H. pylori* eradication is a treatment used for cITP in patients with *H. pylori*. The effect of *H. pylori* on the platelet count of non-cITP individuals has not been previously investigated in New Zealand (NZ). This study sought to establish if *H. pylori* infection decreased the platelet count among patients attending endoscopy clinic at Middlemore Hospital (MMH) and the Manukau Surgical Centre (MSC) in the South Auckland region of NZ. The study also investigated whether infection with cytotoxin–associated gene product A (cagA) positive strains of *H. pylori* had a greater effect on the platelet count compared to cagA negative infections and whether eradication therapy was associated with an increase in the platelet count.

Methods: Patients referred for gastro-endoscopy to MMH and the MSC were recruited for this study. Those who were bleeding, pregnant or had been diagnosed with a disorder likely to affect the platelet count were excluded. Gastric biopsy samples were screened using the Campylobacter-like organism test (CLO-test) for *H. pylori* infection and examined histologically for infection. Full blood counts (FBC) were performed on all patients and cagA serology on *H. pylori* confirmed cases. Biopsy positive, *H. pylori* infected patients were treated with antibiotics and followed up with stool sample screening using the *H. pylori* stool antigen test. The platelet counts from both infected and non-infected groups, and from patients post eradication therapy, were evaluated using the Kolmogorov-Smirnov test (KS-test).

Results: Of the 165 patients enrolled in the study, 24 were CLO-test positive for *H. pylori* infection. The mean platelet counts for the *H. pylori* infected and uninfected groups were 247 and 282 x 10^9/L respectively. Of the infected group, cagA positive and cagA negative infections gave a mean platelet count of 237 and 261 x 10^9/L respectively. The mean platelet count for those undergoing eradication treatment was 258 x 10^9/L which increased to a mean of 268 x 10^9/L post treatment.

Conclusions: This study showed some difference between the mean platelet counts of *H. pylori* infected and uninfected patients (p = 0.0473). Patients infected with cagA positive strains, tended to have a lower mean platelet count compared to those with cagA negative strains. Eradication of *H. pylori* infection failed to make a significant difference to the platelet count in this study.

Keywords: *Helicobacter pylori*, thrombocytopenia, immune thrombocytopenia purpura, eradication, platelet counts.

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INTRODUCTION

H. pylori are fastidious, microaerophilic, helical shaped, Gram-negative bacteria. During infection, flagellum-associated proteins and adhesins facilitate the adherence of *H. pylori* to gastric epithelial cells [1,2]. *H. pylori* produce lipopolysaccharide O-antigen units that can post-translationally be fucosylated to generate Lewis (Le) antigenic structures. The *H. pylori* strains can be classified as types I and II, depending on the expression of the cytotoxin associated gene product A (cagA) and vacuolating cytotoxin A gene product (vacA).

H. pylori infection has been implicated in chronic immune thrombocytopenia (cITP), rheumatoid arthritis and autoimmune thyroiditis [5-9]. Chronic ITP affects mostly adults with thrombocytopenia lasting usually for at least 12 months. Acute ITP normally lasts less than six months and is more common among children. Both disorders can be characterised by a purpuric rash, an increased tendency to bleed. Both conditions caused by increased platelet destruction in which anti-platelet antibodies sensitise circulating platelets marking them for removal by splenic macrophages bearing Fcγ receptors.

Some studies have proposed an association between *H. pylori* infection and ITP and in some cases infection eradication has returned the platelet count within the reference range. This has been especially the case in studies reported from Japan and Italy [10-13]. Just how *H. pylori* infection leads to cITP in some is not known. Approximately 90% of Japanese and East Asian strains and 60% of isolates from the Western world express the cagA virulence protein [8]. The cagA protein has been reported to be expressed in some *H. pylori* infected cITP patients and it has been proposed that anti-cagA might cross-react with platelet membrane glycoproteins [14]. Another proposed mechanism follows P-selectin-dependent platelet aggregation induced by *H. pylori*. Antibodies form a complex with the platelet FcγRIIA receptor leading to platelet activation and the release of P-selectin and von Willebrand factor which promotes platelet aggregation and apoptosis [15]. In the third proposed mechanism, *H. pylori* could invoke an IR producing antibody with Lewis blood group system specificity. In these examples it has been proposed that anti-Le^y auto-antibody promotes adhesion of *H. pylori* to the gastric epithelium leading to atrophy and secondary binding to circulating platelets in those with an appropriate genetic background [8, 16].

The effect of eradication of *H. pylori* and the normalisation of the platelet count in cITP patients was first reported by Gasbarrini *et al*. in 1998 [11]. In that study *H. pylori* eradication led to an increase in the mean platelet count from 75 to 160 x 10^9/L ($p < 0.05$) in eight cITP patients [11]. Similar studies reported more variable platelet count responses with possible causes reported to be related to environmental factors, differing geographical regions, the genetics of the host and *H. pylori* strain variation [2, 10, 17].

Little work into the association between *H. pylori* infection and the platelet count in non cITP patients has been conducted. This study investigated whether the platelet counts of people with *H. pylori* infection were lower than those without the infection. In addition the study sought to establish whether infection with cagA positive strains affected the platelet count more than cagA negative strain infections. The study also investigated whether eradication of *H. pylori* correlated with an increase in the platelet count.

MATERIALS AND METHODS

Patient recruitment

Patients referred for gastro-endoscopy to Middlemore Hospital (MMH) and the Manukau Surgical Centre (MSC) in South Auckland between the 7th of August 2012 and 30th of July 2013 were invited to participate in the study. Patients taking medication likely to affect the platelet count were excluded as were cancer patients and those with infectious, haematological or immunological related medical conditions. Patients below the age of 16 and those mentally unfit to provide informed consent were also not accepted. Patients who had been admissions to hospital in the previous two months, those with a history of transfusion within the previous three months and women who were pregnant were also excluded.

Approximately 500 patients who visited the MMH and MSC for gastro-endoscopy were interviewed for the research and 180, who met the acceptance criteria, were enrolled in the study over a 12 month period. Seventeen patients were later excluded after a review of their recent laboratory data. From the 163 patients, 24 were confirmed to be infected with *H. pylori* by CLO testing and/or histological biopsy.

CLO test

All patients underwent gastro-endoscopy and biopsy samples taken by the gastro-endoscopy team from the antrum of the patient's stomach. Biopsied tissue was used for the Kimberly-Clark CLO-test and histological examination. Tissue immersed in the CLO-test urea gel medium was left at room temperature for 4 to 24 hours. The presence of the urease enzyme produced by *H. pylori*, hydrolyses urea to ammonia, increasing the pH causing a colour change of the medium from yellow (negative) to red (positive).

Histology

Gastric biopsy specimens were processed at MMH laboratory. Samples were first fixed, embedded into tissue sections, cut and then stained with haematoxylin & eosin (H&E). Samples positive by H&E were confirmed by Giemsa staining.

Platelet counts

Ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood was collected by venipuncture from patients at the time of tissue biopsy collection. Platelet counts were performed at the MMH laboratory using a Sysmex XE5000 Haematology Analyser. The platelet count reference range for the region served by the Counties Manukau DHB population had been previously established from population studies. A mean platelet count of 275 x 10^9/L ± 2 standard deviations (SD) provided a platelet reference range of 150-400 x 10^9/L with a confidence interval of 95%.

CagA immunoblots

Serum from CLO-test positive patients was used for the immunoblot method (*H. pylori* - IgG ViraStripe) for anti-cagA. The presence of plasma anti-cagA and a positive immunoblot indicated infection with a cagA positive strain of *H. pylori*.

H. pylori stool antigen test

The Immunocard (Meridian Bioscience Inc.) Helicobacter Pylori Stool Antigen (HPSA) test was used to detect *H. pylori* antigens in the faeces of infected patients following eradication therapy.

H. pylori eradication treatment

H. pylori infected patients were prescribed the standard *H. pylori* triple antibiotic therapy regimen of amoxicillin, omeprazole and clarithromycin for one to two weeks.

Statistical analysis

The platelet count mean, median, ranges, 25th percentiles, 75th percentiles, and the lower and upper 95% confidence limits for infected and uninfected groups were calculated. The Kolmogorov-Smirnov test (KS-test) was used to compare the platelet counts of the two groups and a *p*-value of <0.05 used to indicate statistical significance. The KS-test was also used to compare the cumulative distributions of pre and post eradication platelet counts for the infected subgroups (anti-cagA positive and negative patients).

The biological significance of the difference in platelet counts among infected and uninfected, anti-cagA positive and negative groups was also assessed according to the ISO Manual for Laboratory Reference Ranges in Different Biochemical Analytes [17]. Based on the manual the range of the platelet counts for both the test and control groups would be considered biologically different if the larger standard deviation was at least 1.5 times or greater than the smaller standard deviation [18]. Small subgroups of data lacking sufficient robustness for analysis were not analysed for statistical significance.

Validity rules

1. A significantly lower ($p < 0.05$) mean platelet count in *H. pylori* infected patients as compared to non-infected patients, or an increase in the post-eradication platelet count would demonstrate an association between *H. pylori* infection and the platelet count.
2. A lower mean platelet count (p value < 0.05) in anti-cagA positive patients versus the anti-cagA negative infected patients control group would implicate anti-cagA or cagA protein expression in the reduction of the platelet count in patients.

Ethical approval

This project was approved by the Northern X Regional Ethics Committee. Ref: NTX/12/04/025, the Counties Manukau District Health Board ref: #1227 and the Massey University Human Ethics Committee.

RESULTS

The ranges of the platelet counts for *H. pylori* infected and non-infected patients were 154-394 x 10^9/L and 168-580 x 10^9/L respectively. The mean platelet counts for *H. pylori* infected and non-infected groups were 247 x 10^9/L (SD: 56 x 10^9/L) and 277 x 10^9/L (SD: 68 x 10^9/L) respectively. Approximately 12.5% of infected and 33% of non-infected patients had platelet counts above 300 x 10^9/L; while 37.5% of infected patients and 10% of the non-infected patients had platelet counts of less than 200 x 10^9/L.

Figure 1 shows the distribution and mean of the platelet counts with infected patients showing a lower mean platelet count and platelet count range as compared to non-infected patients.

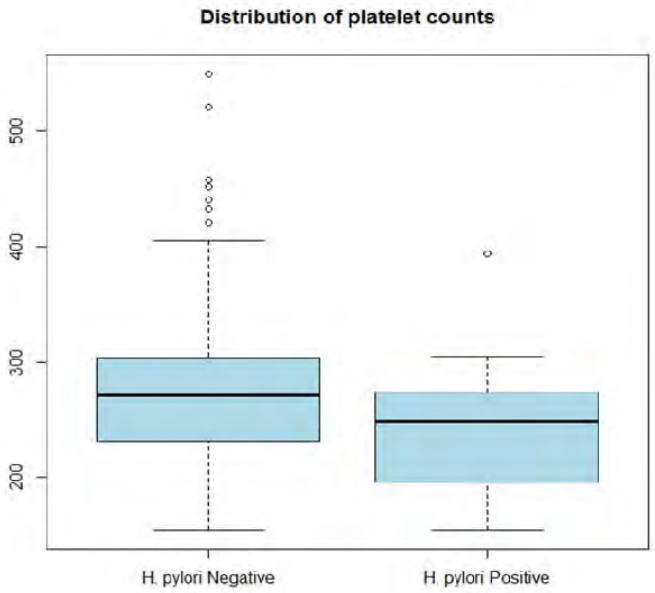

Figure 1. Box plots for the platelet counts from *H. pylori* infected and uninfected patients**.**

Eighteen *H. pylori* participants were tested for serum anti-cagA and eight patients were positive. The mean platelet counts for cagA-positive infections was 237 x 10^9/L (SD: 37 x 10^9/L; range: 190 - 298) and for cagA negative infections, 261 x 10^9/L (SD: 69 x 10^9/L; range: 154 - 394). Figure 2 presents the box plot results of the platelet counts for the three patient groups: uninfected, infected (cagA neg) and infected (cagA pos). The SD of both cagA negative and the non-infected group was 1.8 fold of the SD for the cagA positive group. This indicates a biologically significant difference in the platelet counts of the anti-cagA positive group compared to anti-cagA negative or non - infected patients [17]. Due to the small sample size, the platelet count results from the cagA positive and negative patient groups did not reach statistical significance.

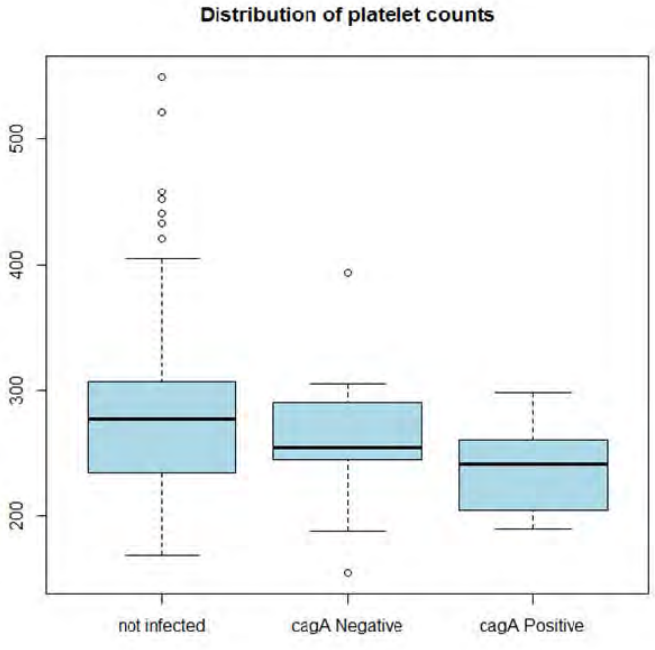

Figure 2. Box-plots displaying the platelet counts for patients not infected, infected with anti-cagA negative and infected with positive strains of *H. pylori*.

The mean platelet counts for anti-cagA positive and negative patients were 237 and 261 x 10^9/L respectively. The range of the platelet counts for cagA negative patients was 154 - 394 x 10^9/L, and the range of the platelet counts for cagA positive patients was 190 - 298 x 10^9/L.

In the follow-up to *H. pylori* eradication treatment, the HPSA test was performed on 20 of the 24 infected patients. Most patients completed treatment but four patients were lost to follow-up and of the remaining twenty; three did not take the prescribed antibiotics. Of the 20 patients, 16 completed the full treatment and tested negative in the HPSA test with one testing positive. Three patients who did not take the *H. pylori* eradication treatment were also positive for *H. pylori* infection. The mean platelet count for anti-cagA positive *H. pylori* infected patients increased slightly from 241 to 254 x 10^9/L following eradication of the infection. For the anti-cagA negative *H. pylori* infected group, the mean platelet count rose from 273 to 287 x 10^9/L following eradication of the infection.

Overall the mean pre and post eradication platelet counts were 258 and 268 x 10^9/L respectively and are shown in Figure 2. Seven patients showed an increase, while eight showed a decrease in the platelet count. The overall results showed an increase in the platelet count of 10 x 10^9/L in fifteen of the previously infected, now infection-free patients.

Table 2. Pre and post eradication platelet counts for cured cagA positive and negative infections.

cagA positive			cagA negative		
Patient	**Pre treatment**	**Post treatment**	**Patient**	**Pre treatment**	**Post treatment**
11	200	325	5	394	369
24	190	179	43	244	243
34	250	236	54	290	362
48	232	235	56	154	158
49	259	254	101	247	312
50	298	321	103	276	232
140	262	231	104	305	333
Mean	241	254	N/A	273	287

DISCUSSION

Approximately 500 patients who visited the MMH and the MSC for gastro-endoscopy were interviewed for the research and 180 who met the acceptance criteria, were enrolled in the study over a 12 month period. Seventeen patients were later excluded after a review of their recent laboratory data. From the 163 patients, 24 were confirmed to be infected with *H.pylori* by CLO testing.

Several hypotheses relating to the platelet count in *H pylori* infected patients, the effect of cagA positive strains on the platelet count and the effect of *H. pylori* eradication on the platelet count were tested in this study. In the first, the hypothesis that the platelet count for *H. pylori* infected patients differed from that of non-infected patients was supported. Results showed a lower overall platelet count in infected patients than in those not infected with *H. pylori* (*p=0.0473*). The second hypothesis tested whether infection with cagA positive *H. pylori* strains was associated with lower platelet counts as compared to cagA negative *H. pylori* strains. This was supported with the SD for the platelet counts from the cagA negative group more than 1.5 times greater than the SD of the cagA positive group.

When the platelet counts of non-infected, cagA positive and cagA negative groups were considered together, the data showed that the mean platelet count and its overall range was higher and wider in the uninfected group as compared to the results from the infected cagA negative group. The infected cagA positive group produced the lowest overall results for the platelet counts. The mean platelet counts for cagA positive and negative patients were 237 and 261 x 10^9/L respectively. The overall spread of the platelet counts for cagA negative patients was 154 - 394 x 10^9/L which was close to the MMH reference range for the platelet count (150-400 x 10^9/L). The highest platelet count for a patient infected with cagA positive *H. pylori* was 298 x 10^9/L. The data showed that infections *H. pylori* expressing cagA protein are associated with a lower platelet count as compared to those infected with cagA negative strains.

The third hypothesis that the platelet counts for infected patients increase after eradication of *H. pylori* infection was tested by repeating the platelet counts of infected patients following *H. pylori* antibiotic eradication treatment. Although the follow up number were few, the mean platelet count for the cured participants increased from 258 to 268 x 10^9/L. The eradication of *H. pylori* infection, irrespective of cagA status, was not associated with an increase in the platelet count with the hypothesis not proven.

The effect of *H. pylori* infection on the platelet count of non-cITP individuals has not been well studied. The studies of Matsukawa et al and Samson et al on non-cITP patients showed little linkage between *H. pylori* infection, the platelet count and eradication of infection [19, 20]. In Australia, Sivapathasingan *et al*., assessed the effectiveness of *H. pylori* eradication therapy on the platelet counts of cITP patients and concluded that *H. pylori* eradication was a possible therapeutic option for treating cITP [21]. Other studies have demonstrated a causal association between *H. pylori* infection and thrombocytopenia in adult cITP patients [13, 22]. According to Campuzano-Maya *et al*, cITP patients can be classified into *H. pylori* dependent cITP, *H. pylori* independent cITP and conventional cITP [8].

In our study the platelet counts for patients with cagA positive versus cagA negative *H. pylori* strains revealed that cagA positive strains may be associated with an overall reduction in the platelet count.

In Japan, *H. pylori* eradication has been used as a treatment for cITP and been shown to be effective at increasing the platelet count in some, while in others there has been no improvement in the platelet count. In our study the mean platelet count for patients cured of *H. pylori* infection increased only marginally by 10 x 10^9/L from the pretreatment count. The mean platelet counts of patients with cagA positive and cagA negative *H. pylori* strains increased by 13 x 10^9/L and 14 x 10^9/L respectively. Eradication of cagA positive *H. pylori* strains was not associated with an increased platelet count; in fact the platelet counts for 50% of the cured patients reduced after eradication treatment.

The platelet count increases seen in some patients after eradication of *H. pylori* in our study should be interpreted with caution as spontaneous platelet count increases without treatment can occur in one third of all childhood and 5% of adult cITP patients [23, 24]. This together with small numbers of untreated and uncured patients limited statistical analysis of some results in our study.

Little information about the effect of *H. pylori* infection on the platelet count among the normal population is available [19, 20]. Our study looked at whether *H. pylori* infection affected the platelet count, whether cagA positive infections had a greater impact on the platelet count than cagA negative strains, and whether *H. pylori* eradication therapy increased the platelet count.

Our study showed a statistically significant difference ($p<0.05$) in the platelet counts of *H. pylori* infected and non-infected patients. This finding receives support from Samson *et al.* who also reported lower platelet counts among *H. pylori* infected patients or subgroups of infected patients as compared to non-infected patients [20]. The mean platelet count difference between infected and non-infected groups in our study showed a seven-fold greater association as compared to that reported by the Samson *et al.* study.

CONCLUSIONS

Although there has been a high prevalence of *H. pylori* sero-positivity (49% in Pacific Islanders) reported in Auckland high school students [4] the prevalence of active *H. pylori* infection in this study was only 14.7% among all enrolled patients. New Zealand Europeans, the majority ethnic group in South Auckland, have been previously reported to have *H. pylori* sero-positivity rates of 14% [7]. More than three quarters of the potential participants in this study were hospital in-patients with advanced illness (e.g. gastrointestinal bleeding) and failed to meet the inclusion criteria and so may have contributed to the low incidence of *H. pylori* infection in this study.

Our study looked to establish whether *H. pylori* infection was associated with low platelet counts, and if so, did cagA strains have a greater impact on the platelet than cagA negative strains. Our study also looked at the effect of *H. pylori* eradication on the peripheral blood platelet count. Patients enrolled in our study were referrals to either the MMH Gastoenterology Clinic or the MSC in South Auckland and biopsy results divided participants into *H. pylori* infected (cagA positive and negative) and non-infected groups. Infected patients were followed up after 3 months to establish if eradication therapy led to an increased peripheral blood platelet count.

Our study showed a tendency for *H. pylori* infected participants to have a mean platelet count lower than that of non-infected patients. No evidence of thrombocytopenia caused by infection was demonstrated in our study. Those infected with cagA positive strains showed a mean platelet count lower than patients infected with non-cagA positive *H. pylori* strains. There was little evidence to suggest that *H. pylori* eradication therapy led to a statistically significant increase in the platelet count.

The sample size for this study was small. Further studies on the effects of *H. pylori* infection on the platelet count of patients in New Zealand, and in particular on cITP patients is indicated by our study.

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ORIGINAL ARTICLE

Evaluation of CHROMagar™mSuperCARBA™ for the detection of carbapenemase-producing Gram-negative organisms

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ABSTRACT

Introduction: Resistance to carbapenems, particularly due to acquired carbapenemases, in Gram-negative bacilli continues to disseminate worldwide. A recent increase in the isolation of carbapenemase producing organisms (CPO) at Canterbury Health Laboratories prompted the need to assess current screening methods. The aim of the study was to evaluate the performance of CHROMagar™mSuperCARBA™ (MSCA) against the current screening protocol of CHROMagar™ ESBL (CESB) and MacConkey agar with a 10 µg meropenem disc (MAC-Mero), for the recovery of CPO in faecal screening samples. We also compared three chromogenic media, MSCA, CESB and ChromID CARBA SMART (IDCA), against a challenge panel of multi-drug resistant organisms (MDRO), in order to determine the sensitivity and specificity of selection.

Methods: 100 screening samples were cultured onto each of MSCA, CESB, and MAC-Mero. Growth of any Gram-negative organism was investigated for the presence of resistance mechanisms. A panel of 50 isolates were inoculated onto each of MSCA, CESB and IDCA. Plates were incubated and examined for growth and typical morphology.

Results: For faecal screening, the specificities of CESB, MAC-Mero and MSCA were 73.9%, 96.6% and 88.6% respectively. No CPO were isolated during the study period. For the MDRO challenge, the respective sensitivity and specificity of each media were as follows: CESB 100%, 5.9%, MSCA 97.0%, 88.2%, and IDCA 90.9%, 88.2%.

Conclusion: The current screening method consisting of CESB in conjunction with MAC-Mero, was found to have the best overall performance. MSCA showed excellent sensitivity and specificity for the recovery of CPO, and would be considered a beneficial addition in an outbreak situation.

Keywords: carbapenemase, chromogenic media, carbapenemase producing organism, screening.

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INTRODUCTION

Resistance to carbapenems, particularly due to acquired carbapenemases, in Enterobacteriaceae and non-glucose-fermenting Gram-negative bacilli continues to relentlessly disseminate worldwide, presenting an enormous challenge to the health sector (1-3). Co-carriage of other resistant mechanisms in these isolates can result in compromised treatment options, and high mortality rates in patients with severe infections (2,4). Carbapenemase-producing organisms (CPO) can reside in faecal flora, posing a risk to the patient of subsequent infections or a potential hazard for patient-to-patient transmission or environmental contamination (5). Early detection of infected or colonised patients is critical to enable effective patient management and to prevent spread of resistant organisms.

New Zealand (NZ) is a country with a low prevalence of carbapenem-resistant Enterobacteriaceae (CRE) and no predominant carbapenemase gene-type (6). With the exception of two recent hospital outbreaks, most cases in NZ have been acquired overseas (6-8). At Canterbury Health Laboratories the first CPO was isolated in 2008, with only three additional isolates over the next seven years. However, in the twelve months preceding this study, fourteen unique CPO were isolated from ten different patients, prompting the need to re-evaluate current screening methods.

There are no published guidelines or 'gold standard' for laboratory screening methods in NZ; however, it is vital that the laboratory use a method which is sensitive enough to detect a variety of CPO but also highly specific in order to reduce unnecessary workup. A range of chromogenic screening media, incorporating antimicrobials, carbohydrates and chromogenic substrates, have been developed for the selective differentiation of carbapenem-resistant Gram-negative bacilli (5).

CHROMagar™ mSuperCARBA™ (MSCA) has recently become available in NZ. MSCA is based on the SUPERCARBA media, but was further developed by CHROMagar™, in conjunction with Dr. Patrice Nordmann, for the detection and isolation of CRE, particularly OXA-48-producers, from clinical samples (9). Carbapenem-resistant Pseudomonas and Acinetobacter are also able to grow on this agar. ChromID CARBA SMART (IDCA), bioMérieux, France, is a bi-plate, consisting of OXA-48-like screening media (chromID OXA-48) on one side and CRE screening media (chromID CARBA) on the other. Both MSCA and IDCA can detect a variety of CPO, making them suitable for use in NZ.

The first part of the study was to evaluate the performance of MSCA against the current screening protocol of CHROMagar™ ESBL (CESB) and MacConkey agar with a 10 µg meropenem disc (MAC-Mero), for the detection of CPO in routine faecal screening samples. The second part of the study was to compare three chromogenic media, MSCA, IDCA and CESB, against a challenge set of multi-drug resistant organisms (MDRO), at low bacterial inoculum, in order to determine the sensitivity and specificity of selection.

METHODS AND MATERIALS

Routine CPO screening

Between April and May 2016, one hundred consecutive screening samples, collected from hospitalised patients, consisting of either faeces or rectal swabs, were cultured onto each of MSCA, CESB and MacConkey Agar with a 10 µg meropenem disc placed in the first quartile. All media was supplied by Fort Richard Laboratories Ltd, NZ. After overnight incubation all colony types growing on MSCA or CESB, or within 25 mm of the meropenem zone, were investigated further. Isolate identification was performed using Bruker MALDI-TOF mass spectrometry (Bruker Daltonics Inc, Billerica, USA). ESBL production was tested for using a double disk synergy test and AmpC production tested for using a combination disk test with cloxacillin, as previously described (10). Pseudomonas or Acinetobacter were tested for susceptibility to meropenem by disc diffusion, whereas Enterobacteriaceae had susceptibility testing performed with the Phoenix™ automated turbidometric growth detection system (BD Diagnostics, Sparks, USA). Interpretation criteria for susceptibility tests were applied according to EUCAST 2016 (The European Committee on Antimicrobial Susceptibility Testing) guidelines and incorporating Phoenix BDXpert rules. Any carbapenem-resistant isolate was investigated for phenotypic carbapenemase production using an in-house CarbaNP test and the carbapenem inhibition method (CIM), as previously described (10,11).

Challenge MDRO

The challenge bacterial panel consisted of fifty non-duplicate multi drug-resistant strains, including 36 Enterobacteriaceae, seven *Pseudomonas aeruginosa* and seven *Acinetobacter baumannii*. Among these isolates there were 33 carbapenemase-producing strains: NDM (*n* = 11), KPC (*n* = 4), VIM (*n* = 3), IMP (*n* = 3), OXA-48-like (*n* = 5, including one strain that coproduced NDM-1), OXA-23 (*n* = 3) and one each of OXA-24, OXA-25, OXA-27, OXA-58; and 17 non-carbapenemase producing isolates that produced other resistant mechanisms such as extended spectrum β-lactamase (ESBL), plasmid-mediated AmpC, AmpC-hyper-production or K1 chromosomal β-lactamase. Six of the 17 non-carbapenemase strains were non-susceptible to one or more carbapenem. All bacterial isolates used in the study were either characterised clinical isolates obtained from Canterbury Health Laboratories or reference strains provided by the Institute of Environmental Science and Research Limited (ESR). *Klebsiella pneumoniae* BAA1705 (KPC positive) and *K. pneumoniae* BAA1706 (KPC negative/ESBL positive) were included in the panel and used as positive and negative controls respectively. Isolates were stored at -80°C and subcultured twice onto blood agar before tests were performed.

To obtain a low-bacterial load, each isolate was suspended in sterile saline to 0.5 McFarland standard, then 20 µl was further diluted into 2 ml of saline. A 10 µl loopful of this suspension was inoculated onto each of MSCA, IDCA and CESB. Plates were incubated at 36°C and examined for growth and typical morphology after 24 hours incubation and again at 48 hours.

RESULTS

Routine CPO screening

In total, 100 screening samples, from 88 patients, were included in the trial comparing the current protocol of CESB and MAC-Mero with MSCA. No CPO were found during the study period, so sensitivity limits were not able to be determined for this part of the study. In total, nine ESBL-producing Enterobacteriaceae were recovered from CESB agar, resulting in a prevalence of 10.2%. CESB grew twenty five non-CPO strains, from 23 patients, resulting in a specificity of 73.9% for the detection of CPO (Table 1).

Table 1. Gram-negative organisms recovered on screening media from clinical samples.

Organism	Resistance mechanism	CESB[1]	MAC-Mero[2]	MSCA[3]	MER[4]	ERT[5]	CarbaNP and CIM
E. coli	ESBL	8	0	0	S	S	
K. pneumoniae	ESBL	1	0	0	S	S	
E. aerogenes	AmpC	1	1	1	R	R	-
E. aerogenes	AmpC	1	0	0	S	S	
E. cloacae	AmpC	1	0	1	S	R	-
E. coli	AmpC	1	0	1	S	S	
Citrobacter spp	AmpC	3	0	0	S	S	
P. aeruginosa	Porin/efflux	5	0	5	S	S	
A. baumannii	Intrinsic	2	0	2	S	NA	
S. maltophilia	Intrinsic	2	2	2	NA	NA	
Total		25	3	12			
Specificity		73.9%	96.6%	88.6%			

[1] CESB = CHROMagar™ ESBL; [2] MAC-Mero = MacConkey agar with 10µg meropenem disc; [3] MSCA = CHROMagar™ mSUPER CARBA; [4] MER = meropenem; [5] ERT = ertapenem;
[6] The number of patients whose samples were positive for the resistant organisms were: CESB 23, MAC-MERO 3 and MSCA 10.

Amongst the 25 strains, eight isolates were ESBL-producing *Escherichia coli*, one isolate was an ESBL-producing *K. pneumoniae* and seven isolates were AmpC-hyperproducing Enterobacteriaceae, including three *Enterobacter* species which were resistant to one or more carbapenem, but tested negative for carbapenemase production. In addition, five *P. aeruginosa* and two *A. baumannii* also grew on CESB; all of which were susceptible to meropenem and ESBL negative. Two isolates identified as *Stenotrophomonas maltophilia* were not tested beyond an identification. All of these strains contained resistant mechanisms that would account for growth on CESB medium.

Only three non-CPO were found to be growing within a 25 mm zone on the MAC-Mero screen (96.6% specificity), including a carbapenem-resistant *Enterobacter aerogenes*, and two *S. maltophilia* (Table 1).

MSCA was a more effective inhibitor of non-CPO than CESB, with a specificity of 88.6%. Twelve strains, from 10 patients were isolated, including three AmpC-hyperproducing Enterobacteriaceae, five *P. aeruginosa* and two *A. baumannii* and two *S. maltophilia* (Table 1). None of the nine ESBL-producing Enterobacteriaceae was recovered on MSCA.

Table 2. Performance of CHROMagar ESBL(CESB), CHROMagar mSuperCARBA (MSCA) and chromID CARBASMART (IDCA) for the detection of multi-drug resistant organisms.

			Chromogenic Screening media			
					ChromID CARBA	
Resistance mechanism(s)	**Species**	**n**	**CESB**	**MSCA**	**CARBA**	**OXA-48**
Carbapenemase-producing organisms						
KPC	*K. pneumoniae*	4	+[1]	+	+	-[2]
NDM-1	*K. pneumoniae*	1	+	+	+	-
NDM-1	*P. stuartii*	1	+	+	+[3]	-
NDM-1, ESBL	*P. mirabilis*	1	+	+[3]	-	-
NDM-1, ESBL	*E. coli*	1	+	+	+	-
NDM-1	*E. coli*	2	+	+	+	-
NDM-1	*P. aeruginosa*	1	+	+	+	-
NDM-1	*K. oxytoca*	1	+	+	+	-
NDM-5	*K. pneumoniae*	1	+	+	+	-
NDM-5	*K. oxytoca*	1	+	+	+	-
NDM-5	*E.coli*	1	+	+	+	-
IMP-4	*E. coli*	1	+	-	-	-
IMP-4	*K. pneumoniae*	1	+	+	+	-
IMP-7	*P. aeruginosa*	1	+	+	+	-
VIM-2	*P. aeruginosa*	1	+	+	+	-
VIM-4	*P. aeruginosa*	1	+	+	+	-
VIM-5	*P. aeruginosa*	1	+	+	+	-
OXA-48	*K. pneumoniae*	2	+	+	+	+
OXA-48/NDM-1	*K. pneumoniae*	1	+	+	+	+
OXA-181	*K. pneumoniae*	1	+	+	+	+
OXA-232	*K. pneumoniae*	1	+	+	+	+
OXA-23	*A.baumannii*	1	+	+	-	-
OXA-23	*A.baumannii*	2	+	+	+	+
OXA-24	*A.baumannii*	1	+	+	+	-
OXA-25	*A.baumannii*	1	+	+	+	+
OXA-27	*A.baumannii*	1	+	+	-	+
OXA-58	*A.baumannii*	1	+	+	-	+
Non carbapenemase-producing organisms						
ESBL	*K. pneumoniae*	2	+	-	-	-
ESBL	*E. coli*	4	+	-	-	-
ESBL	*P. mirabilis*	1	+	-	-	-
ESBL	*P. aeruginosa*	1	+	+	+	+
ESBL/AmpC-hyperproducer	*E. coli*	1	+	-	-	-
ESBL/AmpC-hyperproducer	*C. freundii*	1	+	-	-	-
ESBL/AmpC-hyperproducer	*E. cloacae*	1	+	-	-	-
K1	*K. oxytoca*	1	+	-	-	-
ACC-type plasmid AmpC	*P. mirabilis*	1	+[3]	-	-	-
plasmid AmpC	*P. mirabilis*	1	-	-	-	-
AmpC-hyperproducer	*E. cloacae*	1	+	-	-	-
AmpC-hyperproducer	*E. cloacae*	1	+	+	-	-
Porin/efflux	*P. aeruginosa*	1	+	-	+	-
Total		**50**				
Sensitivity for CPO			100%	97.0%	90.9%	
Specificity for CPO			5.9%	88.2%	88.2%	

n = number of strains tested.
[1] + Growth and colour morphology as expected after 24 hours.
[2] – No growth after 48 hours.
[3] + Growth only after 48 hours.

Challenge MDRO

CESB, MSCA and IDCA were compared for sensitivity of detection for 33 CPO and inhibition of 17 non-CPO culture strains (Table 2). CESB was the most sensitive, detecting 33/33 (100%) of the carbapenemase-producing strains. In addition, 11/11 ESBL-producing strains were also detected on CESB. One *Proteus mirabilis* harbouring a plasmid-mediated AmpC failed to grow on CESB after 48 hours and an ACC-producing *P. mirabilis* grew only scanty colonies after 48 hours. All of the study strains would be expected to grow on CESB; however for the purposes of detecting CPO, this media would have a specificity of just 5.9%.

MSCA showed excellent sensitivity, with 31/33 (93.9%) of the carbapenemase-producing organisms growing after 24 hours incubation and all strains displaying colonial morphology as indicated by the manufacturer. A NDM-1-producing *Proteus mirabilis* grew just one colony after 48 hours, bringing the final sensitivity to 97%. An IMP-4-producing *E.coli* failed to grow after 48 hours and repeat testing produced the same result. Of the seventeen non-CPO, one ESBL-producing *P. aeruginosa* and one AmpC- hyperproducing *Enterobacter cloacae* both grew on MSCA (88.2% specificity). Both of these isolates were highly resistant to meropenem (MIC of >32 mg/L and 16 mg/L respectively) but were phenotypically and genetically negative for carbapenemase.

The IDCA bi-plate had the lowest sensitivity for CPO detection, at 90.9%. On the OXA-screen section, all five of the OXA-48-like isolates grew but two of the oxacillinase-producing *A. baumannii* strains failed to grow. The OXA-screen was very specific for oxacillinase producers, inhibiting all of the remaining study strains, except for one ESBL-producing *P. aeruginosa*. On the CARBA section, 27/33 CPO grew after 24 hours (81.8% sensitivity). A NDM-1-producing *Providencia stuartii* grew a few colonies after 48 hours, giving a final sensitivity of 84.8%. Five CPO isolates failed to grow after 48 hours, with repeat testing producing the same results. Of note is that the growth of several study strains tended to be less plentiful on IDCA than growth on MSCA. Among the non-CPO group, only two carbapenem-resistant *P. aeruginosa* grew on the CARBA side, giving a specificity of 88.2%.

DISCUSSION

Rapid and accurate identification of patients colonised with CPO is critical to control the spread of nosocomial infections and to initiate correct antimicrobial therapy. Although current reports of CPO in NZ are rare, most cases have links to overseas hospital care or travel (12,13). Active surveillance of these high-risk patients as well as critical care areas such as ICU and Bone Marrow Transplant Units is prudent in order to prevent CPO becoming endemic in NZ.

There is no 'gold standard' method for surveillance testing. Current molecular methods, used directly on clinical samples, offer sensitive and rapid results, but this technology is expensive, is limited to the detection of known genes and organisms are not available for identification or antimicrobial profiles. Chromogenic screening agars are useful culture-based methods, but have primarily been developed for the recovery of CRE or even for a geographical area with a predominating gene -type e.g. KPC producers in the USA (5), rather than for a diverse range of CPO. The Centers for Disease Control and Prevention has recommended the use of an overnight broth enrichment step for the recovery of CRE, but the slight increase in sensitivity may not be sufficient to outweigh the disadvantage of prolonged time-to-detection or a resulting decrease in specificity (14,15).

At Canterbury Health Laboratories, we consider that the detection of carbapenemase-producing Pseudomonas and Acinetobacter is also important (3). *A. baumannii* strains that harbour oxacillinases may not be a significant infection control concern, as oxacillinases are rarely found in other genera, but there is still potential for nosocomial spread and infection. *P. aeruginosa* is an important nosocomial pathogen, especially in vulnerable patients, and strains can harbour mobile genetic elements (3). CESB is an essential part of our routine screening so that patients who are colonised with ESBL producers are identified. However, most ESBL-type screening media contain cefpodoxime, potentially inhibiting those CPO with low carbapenem MICs and no coproduction of ESBL (9). In addition, media designed for ESBL detection maybe inefficient for CPO recovery in areas that have high prevalence rates of ESBL or AmpC-hyperproducing Enterobacteriaceae (15).

The finding of a 10.2% prevalence rate for ESBL producers in the patient screening study is higher than we would have predicated, based on annual prevalence rates of <4% in clinical isolates. However, our study is small and this rate of ESBL prevalence may be over-estimated. Even so, this rate of ESBL prevalence should not hinder the use of an ESBL-based screening media for the detection of CPO in our current setting.

Our study found that using CESB in conjunction with MAC-Mero would be sensitive for the detection of ESBL producers from patient samples, and would also recognise potential CPO within the meropenem zone. Furthermore, the inclusion of a non-selective agar such as MacConkey has the added benefit of acting as an 'honesty' plate, whereby the growth of faecal flora can confirm adequate sample collection. MSCA also performed well in this part of the study, with the resulting specificity of 88.6% being higher than that found in earlier studies using the original SUPERCARBA formulation (9,16). Of note is that only 3/12 of the non-CPO recovered were Enterobacteriaceae, which would have resulted in a much higher specificity had only detection of CRE been evaluated.

The second part of our study, evaluating MSCA, IDCA and CESB against fifty MDRO, found that CESB had superior sensitivity (100%) for the detection of CPO. However, the ability of non-CPO to grow so well on this media may mask the growth of low numbers of CRE in a clinical setting, or fail to grow CRE that are susceptible to cefpodoxime. In comparison, the final sensitivity of MSCA was 97%, with a specificity of 88.2%, which compares well with earlier SUPERCARBA evaluations, using a diverse range of CRE (9,16,17). IDCA was the least sensitive agar for the recovery of CPO, with a combined sensitivity of 90.9%. This result is similar to that found by Girlich et al when they compared chromID CARBA plus chromID OXA-48 with SUPERCARBA for the detection of OXA-48-like producing Enterobacteriaceae (18).

A Belgium study by Heinrichs *et al.* evaluated chromID CARBA and Brilliance CRE (Oxoid, ThermoFisher), comparing a direct plating method and a MacConkey broth pre-enrichment step, on 730 rectal swabs (19). Their study found a slightly improved sensitivity for the detection of OXA-48-like producing Enterobacteriaceae with the enrichment step, but the 24 hour time-to-detection delay was a disadvantage. In contrast to our finding, they reported that incubation beyond 24 hours did not increase recovery of CRE, but had the added disadvantage of decreased specificity.

Screening of patients for ESBL and CPO colonisation should be part of a package of intervention strategies used to limit the spread of MDRO and appropriate choice of chromogenic media will ultimately depend on local epidemiology, including established MDRO types.

Limitations of this study include the relatively small number of screening samples tested and the lack of any carbapenemase-producing isolates recovered during the study period. We did not test multiple combinations of CPO on a single plate, which could well mimic clinical samples, when comparing the sensitivity and specificity of the three screening media. This may have affected overall performance.

In summary, our study has shown that CESB used in conjunction with MAC-Mero performs with high combined sensitivity and specificity for the detection of ESBL and CPO in the New Zealand setting. MSCA also performed with excellent sensitivity for the detection of CPO and would be a useful addition to the screening strategy during a CPO outbreak or if local MDRO prevalence rates were to significantly increase.

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ORIGINAL ARTICLE

Evaluation of particulate material in stored units of New Zealand re-suspended red cells

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ABSTRACT

Objective: To evaluate the amount and nature of aggregated protein material in units of New Zealand re-suspended red cells (RRC).

Methods: Eleven units of RRC were passed through filtered intravenous administration sets that contained a mesh filter, using a method similar to a standard patient transfusion protocol. The filters were then flushed gently with saline to remove free red cells, inspected for aggregates and agitated gently at 37°C to dissolve trapped proteins. Samples of the flushing saline were collected both before and after the 37°C incubation. All samples were tested for total protein; the sample with the highest protein concentration was run on mass spectrometry to determine the composition of the protein.

Results: Negligible visible aggregated material was observed or detected in the protein assays of flushing solutions. Mass spectrometry identified that the major protein was haemoglobin. This protein is likely to have arisen from residual red cells in an area of the filter which could not be flushed thoroughly.

Conclusions: There was no aggregated material found in this experiment and it can be presumed that current storage procedures and processing to remove leucocytes and coincidentally platelets, satisfactorily prevents formation of aggregated particulates of leucocytes, platelets and fibrinogen. The methods used in this study were not able to confirm whether filter clogging by aggregates remains a bedside issue, although anecdotal information states that a problem still exists. An alternative method that can detect loosely aggregated material unable to withstand the shearing forces of flushing and which may not even withstand rapid transfusion will be required, together with direct study of administration set filters immediately after bedside transfusions.

Keywords: red blood cells, total protein, particulate, protein aggregate, leucodepletion.

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INTRODUCTION

Patients suffering from acute or ongoing haemorrhage, or clinically symptomatic anaemia are transfused with re-suspended red cells (RRC) (1). Preparation of this clinical blood component involves leucocyte depletion by high-efficiency filtration in an adsorption depth filter, followed by a centrifugation step and separation of the red cells and plasma (1). The red cells are then resuspended in saline, adenine, glucose and mannitol (SAGM) additive solution with only a minimal volume of plasma. SAGM maintains the viability of the red cells during 4°C storage and dilutes the red cells allowing them to flow freely through the intravenous (IV) administration sets used to connect the blood component to the patient (2). The specification for residual leucocyte count is less than 1 $x10^6$ /unit. The specification for filter mesh size in the transfusion administration set is 170-200 µm (3).

Prior to the introduction of pre-storage leucocyte depletion, the presence of aggregated material in stored red cell units would frequently result in clogging of the infusion set after several units had been transfused (4). Studies of aggregated material in unfiltered units of blood have indicated that platelets, granulocytes and monocytes, together with small amounts of fibrin, are the principal constituents (5-9). Maximal formation of aggregates occurs after 2-3 weeks storage (7,10), but the mechanisms for formation has not been confirmed at the molecular level. The role of platelets and leucocytes are thought to be essential for formation of the aggregates but it is suspected that thrombin, fibrinogen and globulins are also contributors (11-13).

Early studies on microaggregates started from an apparent association with post transfusion acute lung injury (14,15). However, subsequent studies that used improved clinical support showed that microaggregates in transfused blood were not the major cause for acute respiratory distress syndrome (ARDS). However, quantitative differences in amounts of aggregated material were considered by some authors to explain the discrepancy in findings.

Other factors, such as sepsis, were the major factor for development of ARDS in many patients. Nevertheless, concern existed over the association of aggregated material with febrile transfusion reactions, transmission of cell-associated cytomegalovirus , immunisation by transfused leucocytes resulting in HLA antibodies and refractoriness to platelet transfusion, post-transfusion thrombocytopenia, immunomodulation and potentially other adverse effects (2,16,17).

This study aimed to determine if visible aggregate material were present in stored New Zealand red cell units and whether these could potentially be trapped in the 170-200 micron administration set filters after the passage of one unit of RRC. The study also assessed the composition of any material trapped and later eluted from the filters.

METHODS AND MATERIALS

Re-suspended red cell (RRC) units that had passed their expiry date were evaluated. Units were kept at room temperature for 30 minutes prior to filtration, to replicate transport and handling time prior to transfusion of red cells. All units were between 35 and 42 days from the date of collection. The transfusion administration sets used were Baxter Clearlink FNC3110 (20 drops per mL) with a 200 µm filter and a roller clamp to regulate flow. The administration sets were primed with phosphate buffered saline (PBS), before each unit was filtered.

A predetermined drip rate was calculated from the volume of each unit and the intended filtration time (180-240 minutes) and controlled by the roller clamp. At all stages, the procedure mimicked those of standard transfusion protocols.

Once filtered, the administration set spike was removed from the unit and the line flushed at 3 mL/min with PBS, until visibly clear of red cells. Flushing volumes were recorded. The IV tubing was sealed at two points: 20 cm and 5 cm below the filter, using a microwave tubing welder. A 15 cm tubing segment was collected as the 'pre' sample for residual protein concentration in the flushing solution, whilst the filter remained full of PBS. A photograph of the filter was taken at this point to record any visible particulate material. The 'pre' samples were frozen at -35°C until testing was carried out.

The filters were sealed and agitated in a 37°C water bath for 2 hours to dissolve any cold-insoluble protein (cryoglobulins or other protein with low solubility) (1). Immediately after removal from the water bath, the remaining saline was collected as the 'post' sample by forward flushing and also stored frozen. Another photograph was taken. As no visible aggregates were evident no further steps were taken to solubilise proteins.

All samples were thawed at room temperature for 9 hours prior to testing. Total protein was tested at Canterbury Health Laboratories (CHL) biochemistry using the Biuret method. The highest of these results was used for mass spectrometry as this was the most likely to provide qualitative results. Liquid phase chromatography was used to separate the sample into its component parts and then time of flight mass spectrometry was used to determine the mass characteristics of the components, using equipment at CHL endocrinology.

RESULTS

Specific details and statistical analysis of the filters used in this experiment are shown in Table 1. The samples had very little protein present and this finding is concordant with the photographs, showing no visible particulates in most filters. A 'post' photograph of the filter with the highest total protein result can be seen in Figure 1.

Figure 1. 'Post' photograph of filter 1.

As any protein present was expected to be predominantly haemoglobin (Hb), a Hb control was used for the mass spectrometry. Results presented in Figure 2 show the control in black with the upper (blue) spectrum showing the results of the test sample. The test sample matched very closely the control results with no other proteins of significance detected by mass spectrometry. The 15126 Da is the peak for the alpha chains of haemoglobin and 15867 Da the beta chains of haemoglobin. Smaller peaks indicated by the callipers are alpha and beta chains with other molecules covalently bonded. The two smaller peaks 162 Da to the right of each chain indicated the presence of HbA1c. The widest callipers (613 Da mass difference) identify alpha chains with extra haem groups abnormally bound. The beta chains with 305 Da added are the result of oxidative damage with binding of glutathione (2).

Table 1. Specific details for each filter and statistical analysis.

Unit	Volume (mL)	Age of RRC at filtration (days)	Time to filter (minutes)	Minimum saline to flush (mL)	'Pre' total protein (mg/mL)	'Post' total protein (mg/mL)
1	293	45	192	43.6	0.00	0.49
2	256	45	201	53	0.04	0.29
3	292	46	224	56.8	0.03	0.10
4	276	41	190	54.6	0.04	0.04
5	262	48	189	41.1	0.02	0.17
6	294	48	227	70	0.13	0.16
7	289	48	191	56.2	0.00	0.09
8	273	47	188	47.8	0.03	0.10
9	242	48	199	78.9	0.03	0.08
10	250	47	197	92.8	0.00	0.13
11	334	47	236	70.3	0.00	0.09
Mean	278.27	46.36	203.09	60.46	0.03	0.16
SD	24.83	2.01	16.56	15.11	0.04	0.12
Median	276	47	197	56.2	0.03	0.1
IQR 1	259	45.5	190.5	50.4	0.00	0.09
IQR 3	292.5	48	212.5	70.15	0.04	0.17

IQR= inter quartile range.

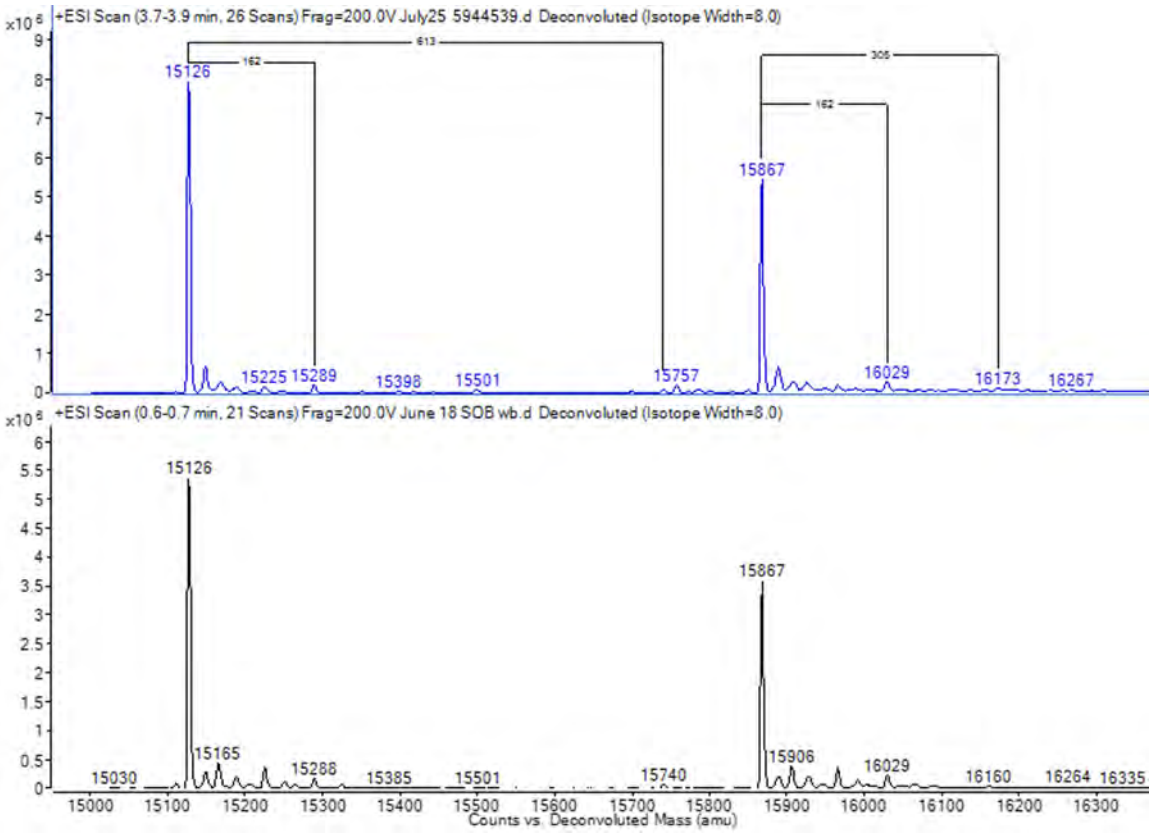

Figure 2. Mass spectrum of 5944539 (upper) and control haemoglobin (lower).

DISCUSSION

This study was performed to investigate quantitatively and qualitatively the presence of aggregated material trapped in the mesh filters of administration sets after passage of one unit of RRC. Published literature is limited despite strong prevalent opinions, that aggregates are a problem among experienced nurses and current transfusion bedside practice guidelines.

Outdated units were used in the study to avoid the use of expensive viable units and also because these have the highest chance of aggregates at or above the expected maximum amount of aggregates (2). Blood banks have refined their operations to minimise wastage of blood components and as a result, few RRC units now reach expiry. A sample number that is larger than the 11 units tested in this study might detect more low frequency events and be more representative of the bedside issues reported by nurses.

Standard transfusion practices for nurses provide rules for changing administration sets. These comply with the guidelines of the Australian and New Zealand Society of Blood Transfusion (ANZSBT) which specify that administration sets must be changed at the end of each transfusion or every 12 hours, whichever comes first (3). The main reason for changing the sets is to minimise the risk of bacterial growth after accidental contamination during connections. This reduces the risk for sepsis in a patient (2).

Trapping of particulate material in the administration sets alters the flow of each unit, and may lead to a blocked filter. Historically, this issue was a well-known cause for slowing of transfusions and difficulty with maintaining flow rates (4,11,12,18). New Zealand Blood Service (NZBS) now employs universal leucodepletion which reduces the content of leucocytes in each unit to 10^{-3} to 10^{-4} of the content in donated whole blood units (1). This practice removes almost all leucocytes from blood components, and reduces the risk of adverse reactions. The most frequent adverse reactions are febrile non-haemolytic transfusion reactions which were reduced by almost 50% after introduction of leucocyte depletion (2).

This step was introduced widely in Europe and later New Zealand after the observation by Aguzzi (reviewed by Turner and Ironside, and by Ponte) that the potential for transmission of variant Creutzfeldt-Jakob disease (vCJD) by leucocytes, and in particular B cells, could be substantially reduced by leucocyte depletion (19,20). Other benefits also exist, notably reduced risk for transmission of cell associated viruses such as cytomegalovirus, and reduced risk of HLA immunisation which may cause refractoriness to platelet transfusion (2). However, leucocyte depletion is thought to be not sufficiently effective to eliminate the risk from graft versus host disease (2).

The leucodepletion procedure involves passing blood through a filter containing tightly compressed fine plastic fibres that have specific chemical epitopes to assist adsorption binding of leucocytes (2). The type of filtration used is termed depth filtration as it relies on a thick layer of fibres over which the cells percolate, rather than screen filtration which has a single membrane or mesh with defined pore size. Aggregates of proteins and cell material are thought to form if leucocytes and platelets remain in stored red cell units and this event may be worst in the buffy coat of leucocytes and platelets that settle on the red cells. Degenerating leucocytes release enzymes that may slowly produce low-grade activation of coagulation and also damage red cells or plasma protein (1,2). This particular type of filtration will eliminate any larger clots from RRC units.

Prior to leucodepletion, units found to completely clog transfusion set mesh filters (17-200 microns) excessively would frequently be sent back to the blood bank for investigation (3). A loose fibrin clot was most commonly at fault in these units. (Faed JM, oral communication, 11 Feb 2016). Degeneration of granulocytes (particularly neutrophils) and monocytes is believed to contribute to particulate development through the release of proteases. Some free proteases are able to activate prothrombin to thrombin and both thrombin and other proteases are able to cleave fibrinopeptides from fibrinogen to form fibrin monomer and fibrin aggregates during storage (1,2).

LIMITATIONS OF THE STUDY

This study had access only to expired (outdated) RRC's but this issue is likely to have led to maximization of the production of aggregates over time, although formation and subsequent dissolving of aggregates either enzymatically or as a result of shearing forces during filtration cannot be excluded. Better control of mixing of blood during collection from the donor and of storage and transport conditions (such as temperature) by the Blood Service may now play a role in reducing the activation of residual leucocytes and formation of particulate material (1).

The use of PBS instead of intravenous grade saline should not have had any significant effect since PBS is isotonic and does not contain ions that may activate coagulation or cells. This is regarded as a negligible difference from standard transfusion protocols (2).

Alternative methods for future studies

Patients often receive multiple red cell units through the same administration set. Comparison of quantities of particulate material after a series of units (e.g. 10 units) that have been passed through a single transfusion set may be more clinically relevant (1,3). Increasing the number of units tested would provide a more representative picture of potential problems with particulates, especially since they now appear to be uncommon following the introduction of leucodepletion (1).

A final recommendation to address several of these problems would be to carry out a retrospective study; collecting used filters from the hospital wards, with relevant information such as number of units transfused and time taken to transfuse. Scientists would then be able to actively assess and investigate issues reported by nurses. This would avoid the potential for laboratory-induced artefacts and differences in the study reported here, when compared with bedside transfusion practice. A retrospective study would require immediate access to the used transfusion sets to evaluate the administration set filters as soon as each transfusion was finished. It would take considerably longer to carry out. Ethics approval would be required if the study involved a change in clinical and bedside practices to facilitate access to administration sets.

CONCLUSION

No particulate material was observed in any of the samples tested in this experiment. This suggests that the procedures in place to reduce the development of aggregates are successful. Nursing staff still believe this is a real problem due to observations with patients. For this reason, further testing is appropriate and should use some of the suggested alterations to the study.

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CASE STUDY

Juvenile myelomonocytic leukaemia and Noonan syndrome: a case study

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ABSTRACT

Both Noonan syndrome and juvenile myelomonocytic leukemia are characterised by hyperactivation of the Ras/MAPK signalling pathway, and as such may manifest concurrently. Here, we report a case study involving twins who originally presented with a petechial rash and a marked thrombocytopenia. Over time, the haematological picture developed into a juvenile myelomonocytic leukemia, and given the rare nature of this disorder, prompted review for other clinical manifestations, thus ultimately allowing the haematologist to consider a syndromic disorder.

Key words: Juvenile myelomonocytic leukaemia, Noonan syndrome, Ras/MAPK signalling pathway.

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INTRODUCTION

Juvenile myelomomonocytic leukaemia (JMML) is a rare but aggressive haematopoetic neoplasm of childhood, classified as a myelodysplastic/ myeloproliferative neoplasm (MDS/MPN) by the World Health Organisation (WHO) (1). The frequency is around 1 per 1.2 million children per year, accounting for only 2% of all childhood leukaemias with a median age of diagnosis of 2 years. JMML is characterised by overproduction of granulocytic and monocytic cells that can infiltrate organs including the spleen, liver, gastrointestinal tract and lung (2).

Features on presentation are non-specific but typically include fever, splenomegaly, cough, rash and a failure to thrive. Haematological findings at presentation can include anaemia, a high white cell count (WBC) with a coexisting monocytosis, thrombocytopenia and an elevated haemoglobin F. A peripheral film examination may demonstrate the presence of immature myeloid and nucleated red blood cells (NRBC) **(**2,3).

The diagnostic criteria for JMML are presented in Table 1. All criteria listed in column A are required, plus at least one from column B or at least two parameters from column C. Sporadic JMML is aggressive and has a poor prognosis (1). The only curative treatment being stem cell transplant, which is only successful in around 52% of patients at 5 years from transplant (2). Failures are often due to graft rejection with relapse or transformation to acute myeloid leukemia (AML) (1).

At the genetic level, 85 – 90 % of JMML patients have a mutation in one or more of the following genes: KRAS, NRAS, PTPN11, NF-1 and c-CBL, all of which are associated with the RAS/MAPK (mitogen-activating protein kinase) signalling pathway, which is involved in regulating cell growth (1,5,6).

As the presenting features for JMML are non-specific, differential diagnoses must be ruled out, including toxoplasma, rubella and CMV. Bone marrow findings in JMML can also be non-specific and atypical for a MPN (1) .

Noonan syndrome (NS), is an autosomal inherited disorder that was first identified in 1962 (5). It is a developmental disorder characterized by a number of features – See Table 2 (5, 7). The frequency is around 1 in 1000-2500 births. Genetic mutations are identified in about 75 % of diagnosed patients, all of which are associated with the RAS/MAPK signaling pathway (7).

We report an apparent case of JMML in twins subsequently diagnosed with Noonan syndrome.

Table 1. Diagnostic criteria for JMML. Table modified from Chang *et al.* (3).

Column A	Column B	Column C
Splenomegaly	Mutations in RAS, PTPN11, NF1 or CBL	WBC > 10 x 10^9/l
Monocytes > 1 x 10^9/L	NF1 diagnosed	Myeloid precursors in peripheral blood
<20% blasts in BM and blood	Monosomy 7	Increased HbF for age
Absence of BCR/ABL fusion gene		Clonal abnormality (other than Monosomy 7)
		GM-CSF hypersensitivity

Table 2. Clinical features of Noonan syndrome (adapted from Tartaglia *et al*. ref. 7).

Facial dysmorphism:	
	Epicanthal folds
	Ptosis
	Down-slanting palpebral fissures
	Triangular facies
	Low set and/or posteriorly rotated ears
	Light coloured irises
Ophthalmologic:	
	Strabismus
	Myopia
Hearing loss	
Dental/oral:	
	Malocclusion
	High arched palate
Skeletal	
	Spinal deformities
	Pectus
	Cubitus Valgus
Lymphatic dysplasias	

(continued overleaf)

Webbed neck with low posterior hairline	
Feeding difficulties	
Cardiovascular:	
	Pulmonic stenosis
	Atrioventricular septal defects
	Aortic coarctation
	Atrial septal defects
	Mitral valve defects
	Ventricular septal defects
	Hypertrophic cardiomyoapthy
Postnatally reduced growth	
Developmental Delay:	
	Attention deficit/hyperactivity disorder
Haematological:	
	Bleeding diathesis
	Thrombocytopenia
	Leukemia

CASE REPORT

Twin A and Twin B were born at nearly 36 weeks, with an Apgar score of 9 and 10 respectively. Both were jaundiced and received phototherapy for the first two days. At 2 days old, a complete blood count (CBC) carried out on both patients revealed that twin A had a marked thrombocytopenia, with a platelet count of 22 x 10^9/L, whilst Twin B had anaemia and a marked thrombocytopenia with an Haemoglobin (Hb) of 128 g/L and platelet count of 26 x 10^9/L (reference range: 145-225 g and 150-400 x 10^9 respectively). Subsequent referred tests demonstrated that the thrombocytopenia was not caused by an immune reaction (e.g. due to incompatibility between mother and baby).

By the end of day three, twin A had developed pancytopenia with a monocytosis, with Twin B also demonstrating a pancytopenia (Table 3). Morphological examination of the peripheral film showed an occasional myelocyte for Twin A. Twin B was transfused platelets. Clinicians were now aware that both patients had a significant heart murmur and likely pulmonary hypertension. At 3 days of age, a toxoplasma screen came back negative, with rubella ruled out at day 5. The CMV screen completed by day 9 also proved negative.

Table 3. CBC results for twin A and twin B at day 3.

	Twin A	Twin B	Reference range
WBC x 10^9/L	8.5	7.9	9.4 – 34.0
Hb g/L	126	120	145 – 225
Platelets x 10^9/L	24	13	150 – 400
Neutrophils x 10^9/L	3.2	3.6	5.0 – 26.4
Monocytes x 10^9/L	2.1	1.5	0.5 – 1.7

By day 8 monocytosis was present in both twins, and by week 5, the changes had markedly progressed (Table 4) with the consultant haematologist describing a '*frank leucoerythroblastic change with circulating blasts*'. A recommendation was made for a bone marrow examination, and the twins were referred to the paediatric haematologist at Starship Children's Hospital. Twin B had now failed an audiology test due to persistent middle ear dysfunction.

A presumptive diagnosis of JMML was made from the haematology results and peripheral film, with a recommendation for a bone marrow examination at 3 months of age once the twins had stabilised/grown. At 14 weeks, splenomegaly was now apparent and the HbF was raised in both twins (38% in twin A and 38.2% in twin B; normal range 10 -35%). A CBC (Table 5), peripheral film and bone marrow was completed on both twins at this time with the peripheral film illustrating nucleated red blood cells, a myeloid left shift including blast cells, but less than 20%.

Table 4. CBC results for twin A and twin B at week 5.

	Twin A	Twin B	Reference range
WBC	16.9	14.3	5.0 – 21.0
Hb g/L	90	96	93 – 158
Platelets x 10^9/L	32	36	150 – 650
Neutrophils x 10^9/L	2.7	3.0	0.5 – 3.9
Monocytes x 10^9/L	2.5	1.7	0.5 – 1.7
Myelocytes x 10^9/L	0.0	0.3	
Promyelocytes x 10^9/L	0.2	0.1	
Blasts x 10^9/L	0.3	0.1	
NRBC x 10^9/L	0.6	0.3	

The bone marrow examination showed an increase in granulopoiesis with around 5% blasts and promonocytes at 7%. Mature monocytes were 5 and 4% (twin A and twin B respectively) and markedly reduced megakaryocytes. Molecular and cytogentic studies were normal confirming absence of BCR/ABL and the consensus criteria for diagnosis of JMML was now met (Table 1).

With JMML confirmed, the attention turned to the diagnosis of an associated syndrome. While JMML is rare, it can at times be associated with an underlying syndrome. The presence or absence of this has a profound influence on the natural history and treatment required so in all cases consideration should be given to careful examination for additional patient abnormalities. In this case this prompting led to the diagnosis of Noonan syndrome based on the patient features – see the discussion below.

Table 5. CBC results for twin A and twin B at 3 months (courtesy of Starship Hospital).

	Twin A	Twin B	Reference range
WBC x 10^9/L	30.1	25.1	6.6 – 15.0
Hb g/L	104	112	97 – 130
Platelets x 10^9/L	39	31	150 – 650
Neutrophils x 10^9/L	11.7	8.6	0.8 – 4.7
Monocytes x 10^9/L	3.3	1.8	0.4 – 1.3
Myelocytes %	13	8.3	
Promyelocytes %	9	9.7	
Blasts %	4.7	4.3	
NRBC %	22.7	25.3	

DISCUSSION

JMML is a rare childhood myeloid neoplasm whose clinical manifestations are heterogeneous and often non-specific. Noonan syndrome is a multisystem developmental disorder characterised by a wide range of clinical features. Both NS and JMML are characterised by hyperactivation of the Ras/MAPK signalling pathway, and as such may manifest concurrently.

Although both JMML and Noonan-JMML are indistinguishable in the laboratory, the likely outcome for patients is vastly different. Untreated sporadic JMML is fatal, and even with aggressive intervention only ~60% of haematopoetic stem cell transplant recipients remain event free after 5 years; the post-transplant relapse rate is high (5, 6). On the other hand, Noonan-JMML usually presents within the months of life and if observed, will spontaneously regress, typically by 1 year of age, without specific therapy (4).

Initially, both twins presented with a marked thrombocytopenia, followed swiftly by the detection of cardiac anomalies. However, it was the deterioration of the laboratory CBC and morphology results indicating a JMML that prompted the consideration of Noonan syndrome. Given the rarity of the JMML leukaemia and the young age of presentation, the possibility a syndromic form of JMML was considered. Clinical examination revealed an upturned nose with flat nasal bridge, wide set eyes with skin folds, dry skin, disproportionately large head and cardiac anomalies, including pulmonary stenosis. While the twins did not have detectable abnormalities in the Ras-MAPK pathway, which are absent in up to a third of cases, the diagnosis of Noonan syndrome or Noonan-like syndrome disorder was established clinically. Despite the haematological findings being indistinguishable from *de novo* JMML, an almost uniformly fatal condition, the haematologist elected to observe for spontaneous remission, given the clinical findings. Serial blood counts showed slow improvement until ultimately the monocytosis, cytopenias and left shifted leucocytosis resolved in both twins by one year of age.

As well as JMML, another example of a childhood haematological syndrome that can spontaneously resolve is transient abnormal myelopoiesis (TAM), a clonal, neonatal myeloproliferative disorder associated with Down syndrome (DS). (9**)** TAM is associated with both trisomy 21 and acquired somatic mutations in the haematopoietic transcription factor, GATA1. TAM frequency is estimated at approximately 10% of neonatal infants with DS (10). Common presenting haematological features include leucocytosis, neutrophilia and thrombocytopenia, with the latter often demonstrating abnormal platelet morphology (9). A blast count of >20% in a peripheral blood film of a DS patient is diagnostic of TAM, whereas blast counts below this threshold would require a GATA1 mutation analysis (9, 10). Blasts most commonly have megakaryoblastic features. Unlike JMML, there is no consistent monocytosis and features are more typical of an AML rather than a MPN. Regression predominantly occurs in the first 3 months of life.

Although a transient haematological disorder, around 20% of infants with DS and history of TAM will develop acute myeloid leukaemia (AML), usually by the age of 4 (11). A prospective study by Massey *et al.* (11) followed 47 DS neonates with trisomy 21 up to the age of 3 months, who had circulating blasts in their peripheral blood/effusions or organ infiltrates over a 5 year period and highlighted some interesting statistics. The mean age for TAM diagnosis was 13 days (range: 1 – 65 days), with the most common clinical features at presentation being hepatosplenomegaly, effusions, bleeding and petechiae. The CBC of 83% of patients (n=35/42) had normalised by a mean of 84 days (range: 2 – 201 days), with 19 % patients developing a subsequent leukaemia (9/47). Both twins were negative for trisomy 21.

The only distinguishing features from JMML and Noonan JMML are clinical manifestations of Noonan syndrome that might alert the clinician. These can be very subtle, as in this case, and as such, can be easily missed. Thus, whenever an apparent JMML presents, it is important to consider the possibility of these associated disorders. While the long term sequelae are not entirely known due to the rarity of the condition, unlike in DS associated TAM recurrence of JMML seems rare. Nonetheless, there are rare reports of additional second malignancies, and as such, careful, long-term follow-up is required.

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HISTORICAL ARTICLE

A retrospective look at clinical biochemistry in 1957

Michael Legge
University of Otago, Dunedin

INTRODUCTION

In 1957 there were no analyzers, most of the equipment was made 'in-house' and often relied on knowledge being 'handed down' and mouth pipetting was the norm. Although a few textbooks were available they were very limited in their methodologies and interpretation of tests. Clinically the range of routine biochemistry tests was limited and the interpretation was similarly limited. The majority of 'routine' biochemistry tests evolved from a chemistry origin and was published in mainly chemistry-orientated journals. The routine pathology laboratory would not normally have access to the majority of these journals and frequently relied on a single textbook for biochemical analysis with the methods being transcribed on to laboratory method cards. It is important to consider that at the time the concept of requesting tests e.g. liver function tests (LFTs) or electrolytes was not known and individual tests were most frequently requested depending on the clinical decision. Most individual tests used one to two ml of either serum or plasma. In this article a retrospective look at the some important biochemical methods available in 1957 and their interpretation is presented with the principles of the methods. For ease of considering the use of tests in a modern context, they have been groups in to a modern view of their use.

Electrolytes

Although the importance of plasma sodium was understood, the relevance of potassium was doubted, as it was primarily intracellular and therefore of 'academic' interest only. Sodium was of clinical interest as it had been recently described as being low in Addison's Disease (Loeb, 1932). A linkage with chloride and bicarbonate was considered and the use of sodium, potassium and chloride were considered 'useful' in surgical emergencies.

Analytically flame photometry was still in its infancy and the chemical method of Trinder's was most frequently used (Trinder 1951). Briefly, sodium was precipitated with magnesium uranyl acetate as sodium uranyl acetate and proteins precipitated with alcohol. Excess uranyl acetate in the supernatant was read against a blank and calculated using sodium standards. This was a modification of the method by McCance and Shipp (1931) who precipitated proteins using tricholoracetic acid and then precipitated the supernatant sodium as sodium zinc uranyl acetate. The precipitate was treated with uranyl ferricyanide and a plum-red colour (uranyl ferricyanide) was compared using a colorimeter against a blank and sodium standards. Results for both methods were reported as mgm/100ml. Potassium similarly relied on a precipitation method but was precipitated directly from the serum using sodium cobalt nitrate giving potassium sodium nitrate, which was washed then dissolved in hot water and choline chloride and sodium ferricyanide added with a resulting emerald green colour, which was proportional to the concentration of cobalt and hence potassium (Jacobs and Hoffman, 1931). Results were calculated using standards and a blank and reported as mgm/100ml. Blood chloride was not considered important unless it was used in conjunction with sodium to establish how much sodium chloride solution should be given in treating burns, vomiting and severe diarrhea. When assayed it was usually using a titrimetric technique such as the one summarized here (Whithorn, 1921). Blood proteins were precipitated using sodium tungstate and sulphuric acid and the chloride in the supernatant precipitated using silver nitrate. Excess silver nitrate was back titrated using potassium thiocyanate with iron-alum as the indicator and calculated as either mgm/100ml of chloride or mgm/100ml sodium chloride.

Liver function tests

In 1957 the concept of using a set of analytes and enzymes was not known and tests of 'liver efficiency' were most frequently used often as single tests. Typically the most common tests were: bilirubin, alkaline phosphatase, zinc sulphate turbidity test and bromosulphalein test. Of these, bilirubin was considered an important analyte as there was a good understanding of jaundice. Urinary bile salt detection (Fuchet's test) was a commonly used technique as it was widely believed that they were formed in the liver, therefore an efficient test of 'liver efficiency' (Kawerau,1953).

Bilirubin

The most commonly used method was that of Van den Bergh (1916), which used diazo reagent (two solutions: A, comprising of sulphanilic acid, concentrated hydrochloric acid and distilled water; B, sodium nitrate and distilled water). These were mixed 1:3 of A and B on the day of the test. Serum was added to the diazo reagent and the colour allowed to develop. If there was no colour within 30 minutes the direct bilirubin was negative. For total bilirubin, serum was added to diazo reagent then alcohol and saturated ammonium sulphate added. The resulting flocculent was centrifuged and the colour of the supernatant compared with a set of cobalt sulphate standards using a Lovibond comparator to give mgm/100ml bilirubin. Each test i.e. direct and total bilirubin required one ml of serum.

Alkaline phosphatase

At the time the origin of alkaline phosphatase was unknown and was thought to be 'leakage' from bones or similar tissues. Elevation of activity was established in obstructive jaundice and bone disease as well as diseases of the liver (Kawerau, 1953). An assay in common use (King and Armstrong, 1934) had serum added to phenyl phosphate (substrate) in an alkaline buffer and incubated with a control which had the proteins precipitated prior to substrate addition. After incubation a phenol reagent (Folin and Ciocalteux reagent) was added to test, control standard and blank, mixed centrifuged and sodium bicarbonate added to the supernatant and the resulting colour read in a colorimeter.

Zinc sulphate turbidity test

Zinc sulphate in a barbitone buffer will precipitate gamma-globulin and was used to establish whether protein electrophoresis was necessary (Kawerau, 1953). The resulting turbidity was compared with a series of protein standards treated identically (10mgm protein equaled one turbidity unit).

Bromosulphthalein (BSP) test

This was an assessment of the liver's ability to clear a foreign substance. A known dose of BSP was injected in to a vein. At 30 minutes post injection, blood was taken from the opposite arm and serum put in to test tubes. One or two drops of sodium hydroxide were added to the first tube and water to the second tube. Any remaining BSP would colour red and could be compared to BSP standards. Normally BSP is cleared within 30 minutes and BSP retention (high colour) was taken to indicate liver disease (Kawerau, 1953).

Pancreatic efficiency

Amylase was a well-recognized test of pancreatic function, although urine amylase appeared to be preferred over blood amylase. Irrespective of whether urine or blood was used the most frequently used method was that based on Wohlgemuth's method whereby starch was digested by urine or blood amylase and the remaining undigested starch was detected with iodine solution. The amylase activity was calculated in arbitrary units (Wohlgemuth's units) relating to the colour development from the addition of the iodine solution (Stocks, 1914)

Renal efficiency

Generally renal efficiency investigations would be triggered by the presence of protein in the urine as well as other indicators such as cells and casts. If the urine protein screen was negative then it was regarded as having no further need to proceed with blood urea or other tests of renal function such as concentration test (McLean and de Wesslow, 1920).

Blood urea

The urease-nesslerization method progressively became the method of choice over other methods (Archer and Robb, 1925). Blood was incubated with a urease suspension (this was usually 'a knifepoint' [0.1g] of soya bean meal), following incubation; proteins were precipitated using sodium tungstate and sulphuric acid. After centrifugation Nessler's reagent (a solution of potassium and mercuric iodide in sodium hydroxide) was added to the supernatant, standard and blank and the resulting colour read in a colorimeter. The test was highly sensitive to ammonia and could be invalidated if ammonia was present in the laboratory.

Blood creatinine

This was not regarded as being as reliable as blood urea for the general diagnosis of renal disease, however, there is evidence that some clinicians used it as a prognostic aid i.e high creatinine concentrations usually had a poor patient outcome. At the time however, little value was attached to any practical use for blood creatinine assays.

Blood uric acid

This is believed to be the first blood analyte to be determined in 1848 by Sir Archibold Garrod using sheep blood. The most frequent use of uric acid was in the diagnosis of gout and notable increases were observed in leukemia, renal disease, and certain cases of eclampsia. Clinical and radiological examination was considered to be generally more important than uric acid in the diagnosis of gout. Blood proteins were precipitated using sodium tungstate and sulphuric acid (Folin, 1939). Supernatant, standard and blank were prepared and 40 per cent urea added followed by 15 per cent sodium cyanide, then the uric acid reaction solution (mixture of phosphoric acid, water, sodium tungstate and lithium carbonate). After incubation the developed colours were read using a colorimeter and uric acid concentration calculated using the uric standard.

OTHER ROUTINE BLOOD TESTS

Glucose (blood sugar)

Blood glucose assays were well established although they were based on the detection of reducing sugars in the blood (glucose being the principle sugar). The use of the blood sugar tolerance tests was well established for the diagnosis of diabetes and for the monitoring in pregnancies when severe glycosuria was identified. At the time there were a number of methods for glucose measurement. The method of Folin and Wu (1920) was frequently used. Blood was deproteinised with sodium tungstate and sulphuric acid, following deproteinisation the supernatant was mixed with an alkaline copper solution (sodium carbonate, tartaric acid and copper sulphate), standards and blank were prepared also. Following incubation at 100^{o}C the tubes were cooled and phosphomolybdic acid solution added. The developed colour was read in a colorimeter after allowing the carbon dioxide generated by the reaction to be liberated.

Cholesterol

Cholesterol was considered most useful for the diagnosis of xanthomatosis (deposits of cholesterol in skin around the body) and hypothyroidism, otherwise it was not regarded of much use in clinical work. The method of Myers and Wordell (1918) was considered the most appropriate technique at the time. Blood was mixed with plaster of Paris and dried. The dried blood was extracted with chloroform then with acetic anhydride and sulphuric acid. A green colour develops and compared with similarly treated standards and read using a colorimeter.

Acid phosphatase

The use of this enzyme in the diagnosis of prostate cancer was well recognized as was the stability of prostate acid phosphatase in the presence of formalin. Usually the alkaline phosphatase method was used with a change of incubation buffer pH to 4.5, the addition of formalin and the increase in the incubation time from 30 to 60 minutes (Gutman and Gutman, 1938).

Calcium

Calcium was considered important in the diagnosis of tetany and for monitoring parathyroid hormone treatment using parathyroid extracts. Although it was recognized that calcium could be analysed by flame photometry, the method of Kramer and Tisdall (1921,) was frequently used. Calcium was precipitated from serum using saturated ammonium oxalate. After centrifugation the tubes were drained and the precipitate washed with dilute ammonia solution followed by 0.1N sulphuric acid, boiled then titrated immediately after cooling with potassium permanganate until a pale pink colour lasting one minute is achieved. Calcium concentration was calculated from the titration. This method uses two ml of serum per test.

Phosphate

Phosphate analysis was considered most helpful in rickets. Proteins were precipitated using trichloroacetic acid. An aliquot of supernatant was treated with molybdic acid and phosphomolybdate, followed by hydroxyquinone in the presence of sodium sulphate. The resulting blue reaction colour was read in a colorimeter compared with standards and a blank (Briggs, 1922).

Acid base

The ratio of carbonic acid to bicarbonate was well recognized in controlling blood pH. However, the principle causes of acid base disruption were considered to be ketosis via the detection of urinary ketones, renal insufficiency, and fever. The determination of acid base balance was difficult with no suitable pH, PCO_2 and PO_2 electrodes.

Bicarbonate

It was widely considered that bicarbonate gave comparable results for alkali reserve, which was similar to total carbon dioxide. Blood was collected under paraffin oil and stirred thoroughly then centrifuged to obtain plasma under oil. Two 100ml conical flasks were prepared ('Reference' and 'Test'). The 'Reference' contained phenol red (indicator) plasma and physiological saline. In the 'Test' phenol red, plasma and 0.01N hydrochloric acid were added. Both flasks were 'whirled' vigorously and incubated for 30 minutes at 37^{o}C. Physiological

saline was then added to both flasks, and the solutions covered with paraffin oil then titrated with 0.01N sodium hydroxide until the 'Test' colour equaled the 'Reference' colour. Plasma bicarbonate concentration was calculated from the titration (Van Slyke, 1922).

Blood proteins

Measurement of plasma or serum proteins was based on the micro-Kjeldahl technique, however, the biuret method was gradually replacing the micro-Kjeldahl technique due to its simplicity. Total protein was precipitated with trichloroacetic acid and the precipitate was dissolved in sodium hydroxide followed by crystalline copper sulphate solution. The resulting colour was read in a Lovibond Comparator. For albumin, ammonium sulphate was added to either plasma or serum and the solution filtered. The filtrate then had trichloroacetic acid added mixed then treated as for total protein (Howe, 1923; Fine, 1935).

Protein electrophoresis

Protein electrophoresis was not a routine technique and was generally indicated by a positive zinc sulphate turbidity test or proteinuria. Electrophoresis tanks were usually made 'in house' to a common design (Flynn and de Mayo, 1951). A barbitone buffer was used and the support medium was Whatman number 1 filter paper strips. The origin was marked with pencil and serum loaded at the origin then the current applied for 16 to 18 hours. The filter paper strips were then removed, dried and stained (often with brom-phenol blue in alcohol and mercuric chloride), rinsed then dried. Five main bands could be located and interpretation related to well defined clinical conditions (Hardwicke, 1954; Flynn and de Mayo, 1951; Flynn, 1954).

CONCLUSION

Over the last 60 years there have been dramatic developments in laboratory medicine and clinical biochemistry is a sharp reflection of such change. As clinical demands evolved new approaches to diagnostic testing were required such as the development of flame photometry for sodium and potassium and the concept of diagnostic test profiles e.g. electrolytes, liver function tests etc. With the advent of biochemistry analyzers, especially the TechniconÔ continuous flow auto-analysers the single biochemical test taking hours to perform with little or no quality control system rapidly disappeared. Acid-base balance also reflected such changes with the development of gas and pH electrodes providing rapid and accurate patient acid-base status. These changes were reflected in a change in the scientific and medical literature with increasing numbers of journals dedicated to the use of the newer technologies and the ability to obtain detailed biochemical analysis of disease states. The rapid availability of biochemical testing and the deceasing volume of blood and plasma to undertake such tests allowed for improved patient management, accurate diagnosis and significant changes in surgical procedures. Analytical techniques have made significant progress since 1957 and today modern medicine is on the cusp of a new generation of technologies and diagnostic power.

AUTHOR INFORMATION

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Barrie Edwards and Rod Kennedy Scholarship report

Max Reed
Wellington SCL

To be chosen as the recipient of the 2015 Barry Edwards and Rod Kennedy Scholarship was an absolute career highlight for me, for which I am hugely grateful to the NZIMLS. I elected to attend the AACC 2015 Annual Meeting and Clinical Lab Expo at the Georgia World Congress Center in Atlanta, Georgia. This meeting had more than 400 sessions over 5 days from July 26-30th, with additional workshops and satellite meetings held on the Saturday prior to the meeting, as well as the Clinical Lab Expo which showcased over 700 exhibitors, and was attended by 18,000 delegates from around the world.

The meeting itself is made up of Plenary sessions featuring visionaries on the future of healthcare, from clinical practice, research, policy and business forums; symposia sessions presented by experts actively involved in their respective areas of expertise, and composed of multiple lectures with Q&A sessions; short courses which give an intensive coverage of a specific subject in a traditional lecture type setting; brown Bag sessions which are small, interactive round table discussions for 8-10 people with a topic expert; meet the experts sessions which are larger interactive sessions with plenary speakers and other experts; and poster sessions, in which my poster on "Assessing the impact of enzymatic creatinine of eGFR in an elderly outpatient population" was included.

To say that attending a meeting of this size is "daunting" is somewhat understating the situation but because of the different sizes of the various sessions, you quickly start to recognise people and make connections with colleagues from around the world, be that as someone to say hello to in passing, or someone to actively communicate with in the future. My first short course was on "The concepts of method evaluation: verification of manufacturer claims for commercial methods and instruments", moderated by Sten Westgard. Having spent many years using Westgard rules, it was awesome to be able to actually attend a session run by Sten, particularly after having registered my attendance with his father, James Westgard, on arriving at the course. And while there was a large FDA slant to this session, it was a useful tour of the CLSI guidelines associated with method validation, and a review of which statistical tools are most appropriate in particular settings.

My next short course was "The opportunity to integrate the laboratory into patient care through meaningful use of electronic health record", and while the subject may sound a tad dry to many, having worked as the NZ representative on the AACB harmonisation working party, as well as contributing to the national review, modification and update of the NZPOCS code sets, this course was fascinating, covering both SNOMED and LOINC codes, ordering and resulting of tests, with a large focus on the American incentive scheme to encourage laboratories to use standardised code sets, to improve consistency of both ordering and reporting of laboratory tests with the flow-on benefits to patients and referrers.

Tuesday's plenary session was "Art, assassination and America – how transparency disrupts an industry and changes a nation", by Dr Martin Makary from Johns Hopkins University. Dr Makary is a surgeon and New York Times best-selling author, as well as a television medical commentator for NBC and Fox News. He is an international expert in patient safety and was a leader in the United Nations WHO Safe Surgery Saves Lives initiative, which developed a 19 item checklist which is used by the majority of surgical providers around the world to reduce errors and adverse events and increase teamwork and communication during surgery. Dr Makary commented that going to hospital is the 3rd biggest killer of Americans, after cancer and heart disease, and that the reason this is so is due to "variation".

By developing a "standard" of care, the variation of health care decreases, patient care becomes more uniform, patients are safer and patient risk is reduced. He also advocates hospitals publishing their "outcomes" so consumers can choose where to go for their healthcare based on performance.

Coming from a bulk-funding model of laboratory medicine, the session on "Laboratory test utilization: leading the charge towards lab stewardship" was very interesting, particularly with the focus on the contribution of unnecessary lab tests to rising healthcare costs. As this was an interactive session, being able to add a New Zealand perspective to the discussions was both entertaining and challenging for me and for the presenters, who were both encouraging and interested in our funding influenced differences. The restriction of testing based on the presence or absence of clinical information was a discussed strategy, particularly favoured by private laboratory providers, but the presenters at this session were also extremely open about the fact that in their funding structure, they actually employ the clinicians, and so they had even greater control over requesting patterns.

Wednesday's plenary session was entitled "Tackling HIV latency: towards a cure for HIV", by Dr Sharon Levin from Monash University, in Melbourne. While HIV is no longer seen as a death sentence, it is currently a lifelong condition requiring lifelong treatment, and the focus of research is now moving to establishing why and how HIV is able to persist long term on treatment. Although rare, case reports of remission in patients who have ceased treatment, are suggesting that a cure for HIV may soon be an achievable goal.

My final Brown Bag session was chaired by Dr Stephen Wong, departing president of the AACC, and was entitled "Dashboard for clinical pathology/chemistry core lab and outreach". While I was hoping for more "answers" to the questions of how best to display laboratory performance in a user-friendly format, this session gave me pointers on where to look and who to talk to at the Clinical Lab Expo. It also gave me the opportunity to talk to Dr Wong, particularly about the format for future AACC meetings. When registering for the 2015 meeting, plenary and symposia sessions were covered by the registration fee, but short courses and brown bag sessions were paid for individually. As outgoing president, Dr Wong was pleased to share the fact that future meetings will cover all sessions within the registration fee.

The Clinical Lab Expo itself is the largest of its kind in the world, with over 2000 booths. The opportunity to view automation systems from suppliers that we don't see in New Zealand, is always fascinating, but the chance to see the sheer range of products and technology available from around the world in all areas of laboratory medicine from allergies and autoimmune disease to viral genotyping and everything in between, was amazing. As an indication of the sheer size of this exhibition, it took in excess of 40 minutes to walk around the outside of the trade displays without even venturing into the individual avenues of displays.

I cannot recommend the experience of attending an AACC annual meeting and Clinical Lab Expo highly enough, and I would like to thank the families of Barry Edwards and Rod Kennedy, and the NZIMLS Council, once again for making my trip possible. If anyone would like to know more about this meeting, please feel free to contact me at max.reed@wellingtonscl.co.nz

THE Pacific WAY

Greetings to you all.

Courses held at the Centre

Biochemistry: 18 July – 12 August 2016

A biochemistry course was provided by the PPTC in July/ August of this year at its centre in Wellington, and the following four students attended: Mele Teukava from Tonga, Rhomson Nuake from the Solomon's, Ivapene Faumuina-Aiafi from Samoa, and Taupesa Taumoeanga from Tuvalu .

Students and staff: Biochemistry 2016

Microbiology: 5th September – 30th September 2016

Seven students attended the microbiology course: Filimone Fili from Tonga, Anareta Ciusia from Fiji, Virginia Conceicao from East Timor, Rattanak Em and Soheap Oeng from Cambodia , Kiaman Raurenti from Christmas Island, and Newton Banisi from the Solomons.

Students, staff, and guests: Microbiology 2016

Becoming an effective laboratory manager: 3 October – 28 October 2016

Six students attended the laboratory managers course: Angelica Mokahega from Niue , Rabbie Nigel Bero from PNG, Seini Rawasoi and Ashmita Chand from Fiji, Nevio da Costa Sarmento from Timor Leste, and Rosemary Tekoaua from Kiribati.

This course which was introduced by the PPTC in 2016, focused on laboratory managers, charge technicians, and those in leadership roles in medical laboratories. Over the duration of four weeks, participants were coached on how to provide effective and efficient administration of the medical laboratory service, including budget planning and control with responsible financial management, in accordance with institutional assignment of such responsibilities. Other areas of importance that were addressed included the provision of educational programs for laboratory staff, as well as participation in educational programs offered by the PPTC. Participants were taught how to plan and direct research and development appropriate to the facility; how to select and monitor all referral laboratories for quality of service; how to implement the quality management systems, to monitor all work performed in the laboratory to determine that medically reliable data is being generated; how to ensure that there are sufficient qualified personnel with adequate documented training and experience to meet the needs of the laboratory and how to plan and set goals and develop and allocate resources appropriate to the medical environment; how to implement a safe laboratory environment in compliance with good practice and applicable regulations; and how to address any complaint, request, or suggestion from users of laboratory services. Individual case scenario discussions were encouraged and included as group exercises. Tours of Wellington medical laboratories were organised for the participants to experience laboratory management in larger hospitals.

Overseas travel

PPTC staff and consultants have travelled extensively throughout the year and activity in the last few months is listed below:

- 20 June - 1 July: Filipo visited Samoa to provide immunoassay analyser training.
- 18 July - 22 July: Russell visited Samoa to perform an accreditation assessment.
- 18 July - 29 July: Navin visited Vanuatu to carry out an assessment of TB training in Vila, Santo and Tana.
- 1 Aug - 5 Aug: Russell visited Vanuatu to carry out LQMS/ accreditation training .
- 15 Aug - 26 Aug: Navin visited the Solomons to carry out HOD serology training.
- 22 Aug - 26 Aug: Filipo visited Samoa to carry out an accreditation assessment
- 5 Sept - 9 Sept: Yvonne Bird visited Tonga to carry out an accreditation assessment.
- 5 Sept - 16 Sept: Russell and Fuianina Washburn both carried out histology training in the Solomons.
- 26 Sept - 7 Oct: Phil and Filipo visited Tonga to carry out HOD training in haematology and biochemistry respectively.
- 10 Oct - 21 Oct: Navin visited Vanuatu to carry out TB training in Santo.
- 17 Oct - 28 Oct: Phil visited Samoa to carry out HOD training in haematology.
- 24 Oct - 4tNov: Filipo visited Samoa to carry out HOD biochemistry training as well as an accreditation audit.

Tonga's annual three day conference

Both Phil and Filipo were fortunate during their visit to Tonga in September to have been given the opportunity to participate in the annual three day laboratory quality management programme and workshop provided by the laboratory management team for all it's laboratory staff.

Tongan students participating in the conference

Centre based courses for the remainder of 2016

Blood transfusion science: 31 October – 25 November 2016

The PPTC would like to welcome Chris Kendrick back to the PPTC as its blood transfusion specialist. Chris will be providing a four week blood transfusion course at the Centre in November and we are most grateful to him for making the time to share his extensive knowledge and experience with our students.

PPTC training courses in 2017

- Haematology and blood cell morphology 24 April - 19 May 2017
- Biochemistry 19 June - 14 July 2017
- Laboratory quality management Systems 7 August - 1 September 2017
- Microbiology 18 September - 13 October 2017
- Blood transfusion science 30 October - 24 November 2017

For further information contact:
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Website: www.pptc.org.nz.

2016 NZIMLS CALENDAR

Dates may be subject to change

DATE	SEMINARS	CONTACT
12 November 2016	Mortuary SIG Seminar	Bill.little@southerndhb.govt.nz Clive.matthews@northlanddhb.org.nz
DATE	**NZIMLS EXAMINATIONS**	**CONTACT**
07 November	QMLT Examinations	fran@nzimls.org.nz
DATE	**COUNCIL**	**CONTACT**
November 2016	Council Meeting	fran@nzimls.org.nz
DATE	**MEMBERSHIP INFORMATION**	**CONTACT**
January	Membership and CPD enrolment due for renewal	sharon@nzimls.org.nz
January	CPD points for 2016 to be entered before 31 January 2017	cpd@nzimls.org.nz
15 February	Material for the April issue of the Journal to be with the Editor	rob.siebers@otago.ac.nz
17 June	Nomination forms for election of Officers and Remits to be with the Membership	fran@nzimls.org.nz
15 June	Material for the August Journal must be with the Editor	rob.siebers@otago.ac.nz
7 July	Nominations close for election of officers	fran@nzimls.org.nz
27 July	Ballot papers to be with the membership	fran@nzimls.org.nz
3 August	Annual Reports and Balance Sheet to be with the membership	sharon@nzimls.org.nz
10 August	Ballot papers and proxies to be with the Executive Officer	fran@nzimls.org.nz
15 September	Material for the November Journal to be with the Editor	rob.siebers@otago.ac.nz
23/12/16—23/01/17	**Executive Office Closed for Christmas Break**	fran@nzimls.org.nz sharon@nzimls.org.nz

Journal Questionnaire

Below are 10 questions based on articles from the November 2016 issue. Read the articles carefully as most questions require more than one answer.

Answers are to be submitted through the NZIMLS web site. 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.

The site has been developed for use with Microsoft's Internet Explorer web browser. If you are having problems submitting your questionnaire and you are using the Firefox web browser, try resubmitting using Microsoft's Internet Explorer.

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 24th February, 2017. You must get a minimum of eight questions right to obtain five CPD points.

The Editors set the questions but the CPD Coordinator, Jillian Broadbent, marks the answers. Please direct any queries to her at cpd@nzimls.org.nz.

NOVEMBER 2016 JOURNAL QUESTIONNARE

1. At Aotea Pathology, what was their initial protocol for the cultivation and isolation of *Salmonella* and *Shigella* from clinical faecal specimens?
2. Both chronic and acute immune thrombocytopenia are caused by what?
3. *Helicobacter pylori* is what type of bacteria?
4. Co-carriage of other resistant mechanisms in Enterobacteriaceae and non-glucose-fermenting Gram-negative bacilli isolates can result in what?
5. What was the current screening protocol at Canterbury Health Laboratories for the detection of carbapenemase producing organisms in routine faecal screening samples?
6. Preparation of re-suspended red cells for transfusion involves what processes?
7. What are the principal constituents of aggregated material in unfiltered units of blood?
8. What concerns exists over the association of aggregated material with febrile transfusion reactions?
9. Juvenile myelomonocytic leukemia is characterised by what?
10. What non-specific features on presentation of juvenile myelomonocytic leukemia are typically included?

AUGUST 2016 JOURNAL QUESTIONNAIRE ANSWERS

1. Name two (out of three) potential implications the medical laboratory should consider while implementing improvements and updates to the laboratory's quality management system.
   1st relates to the consideration of comfort, engagement, and ergonomics for laboratory personnel. 2nd concerns the level and type of quality training provided by the medical laboratory. 3rd concerns the ability for the medical laboratory to be open to emerging technologies and trends within areas of operation.
2. To determine a true HbA1c result which alternative analytical methods can be used?
   Boronate affinity chromatography, capillary electrophoresis, and mass spectrometry.
3. Name the less common sites of metastases of thyroid carcinoma.
   Brain, liver, kidneys, skin, and breast.
4. What is a good marker in the confirmation of the pathological diagnosis of differentiated thyroid cancer metastasis?
   Anti-thyroglobulin antibody.
5. What is associated with fewer cancer recurrences and tumour related deaths from metastatic thyroid papillary carcinoma to the breast?
   Thyroidectomy, post-operative iodine ablation, thyroxine replacement, and careful follow-up with serum thyroglobulin levels.
6. von Willebrand disease is a heterogeneous bleeding disorder arising from what?
   Either a quantitative or qualitative defect and/or a deficiency in von Willebrand factor.
7. How is von Willebrand disease inherited and what type of mutations can occur in the von Willebrand factor gene?
   As an autosomal disorder. Deletions, frameshift, splice-site, and nonsense mutations.
8. Name the limitations and laboratory problems for the von Willebrand factor ristocetin cofactor assay.
   Difficult to perform, time consuming, poor reproducibility, sensitivity, difficult to interpret, and raises serious concerns in quality assurance surveys.
9. What type of assay is the von Willebrand factor collagen binding assay and what does it measure?
   ELISA. Measures the functional ability of large von Willebrand Factor multimers to bind external collagen.
10. The Innovance von Willebrand factor activity (vWF:Ac) assay can theoretically be compromised by which analytical factors?
    Interference with icteric, lipaemia, rheumatoid factor, heamolysis, and some specific medications

BARRIE EDWARDS & ROD KENNEDY SCHOLARSHIP

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

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

All applications will be considered by a panel consisting of the President and Vice-President of the NZIMLS and the Editor of the New Zealand Journal of Medical Laboratory Science (who are ineligible to apply for the scholarships). The applications will be judged on your professional and academic abilities together with your participation in the profession. The panel's decision is final and no correspondence will be entered into.

Application is by letter. Please address all correspondence to:

NZIMLS Executive Officer
PO Box 505
Rangiora 7440

Barrie Edwards

Rod Kennedy

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

In your application letter please provide the following details:

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

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.

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