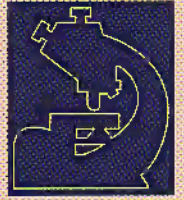


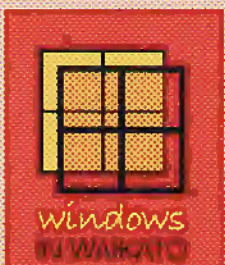
Volume 58 Number 2 August 2004

ISSN 1171-0195



# New Zealand Journal of Medical Laboratory Science

Official Publication of the New Zealand Institute  
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# 2



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The Journal is abstracted by the Cumulative Index to Nursing and Allied Health Literature (CINAHL), Index Copernicus, Excerpta Medica/EMBASE, Chemical Abstracts, and the Australian Medical Index. The journal, through its Editor, is a member of the World Association of Medical Editors (WAME).

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New Zealand Journal of

# Medical Laboratory Science

Volume 58      Number 2  
August              2004  
ISSN 1171-0195

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## Editorial

### Geopolitical intrusion on editorial decisions

Recently, in the USA the Government tried to place a ban on USA-based biomedical journals in publishing articles from various countries for whom the USA had a trade ban with. The editors from these journals contacted WAME (World Association of Medical Editors) for advice as they were concerned about political interference with editorial decisions. A lively discussion followed on the list serve of WAME with a virtual condemnation of such interference. After consultation with its members, WAME put out a policy statement, which is reprinted below.

*"Decisions to edit and publish manuscripts submitted to biomedical journals should be based on characteristics of the manuscripts themselves and how they relate to the journal's purposes and readers. Among these characteristics are importance of the topic, originality, scientific strength, clarity and completeness of written expression, and potential interest to readers. Editors should also take into account whether studies are ethical and whether their publication might cause*

*harm to readers or to the public interest. Editorial decisions should not be affected by the origins of the manuscript, including the nationality, ethnicity, political beliefs, race, or religion of the authors. Decisions to edit and publish should not be determined by the policies of governments or other agencies outside of the journal itself."*

WAME is a voluntary association of editors from many countries who seek to foster international cooperation among editors of peer-reviewed biomedical journals. Both the Editor and Deputy-Editor of the *New Zealand Journal of Medical Laboratory Science* are members of WAME and whole heartedly support the above WAME policy statement.

**Rob Siebers, FNZIMLS, Editor; Ann Thornton, FNZIMLS, Deputy-Editor. Wellington School of Medicine and Health Sciences**

#### Med-Bio Journal Award



Med-Bio offers an award for the best article in each issue of the *New Zealand Journal of Medical Laboratory Science*. All financial members of the NZIMLS are eligible. The article can be an original, review or technical article, a case study or a scientific letter. Excluded are editorials, reports, or fellowship treatises. No application is necessary. The Editor and Deputy Editor will decide which article in each issue is deemed worthy of the award. If in their opinion no article is worthy, then no award will be made. Their decision is final and no correspondence will be entered into.

# Method evaluation for methylmalonic acid: use for assessing vitamin B<sub>12</sub> deficiency

Christine C Leaver, BSc, FNZIMLS;  
Specialist Biochemistry, Canterbury Health Laboratories, Christchurch

## Abstract

**Background:** Vitamin B<sub>12</sub> deficiency is a common cause of macrocytic anaemia and is implicated in a spectrum of neuropsychiatric disorders. Diagnosis is typically based on the measurement of serum vitamin B<sub>12</sub> (B<sub>12</sub>). However a low serum B<sub>12</sub> frequently does not indicate deficiency, and a "normal" serum B<sub>12</sub> does not rule out deficiency. Serum methylmalonic acid (MMA) is elevated in vitamin B<sub>12</sub> deficiency. Holotranscobalamin II (TCII) the active form of B<sub>12</sub>, is thought to be an early indicator for B<sub>12</sub> deficiency.

**Aim:** The study aim was to set-up and evaluate a serum MMA, GCMS method for vitamin B<sub>12</sub> deficiency. To determine the reference range and assess results using a new expensive kitset method for TCII.

**Methods:** After analytical evaluation of the MMA method: MMA, B<sub>12</sub>, TCII were assayed in 158 "normal" adult volunteers. Patient samples (189) requested for B<sub>12</sub> and MMA (52% older than 65 years and 80% with B<sub>12</sub> levels less than 200 pmol/L) were processed, 67 of which were also tested for TCII.

**Results:** In 158 normal volunteers 6 were B<sub>12</sub> deficient (MMA >0.35 µmol/L, TCII <27pmol/L). Out of 58 people with B<sub>12</sub> levels between 150 and 200 pmol/L, 14 had high MMA and 13 (93%) of those had low TCII, the remaining 44 had normal MMA of which 38 (86%) also had normal TCII.

**Conclusions:** The GCMS MMA method was found to be accurate, precise and robust. The reference range was clinically determined to be <0.4 µmol/L. We suggest MMA be tested when B<sub>12</sub> levels are below 200 pmol/L, in patients suspected of having B<sub>12</sub> deficiency, especially when other tests are inconclusive.

**Key words:** Methylmalonic acid, Vitamin B<sub>12</sub> deficiency, Holotranscobalamin II.

## Introduction

Vitamin B<sub>12</sub> (B<sub>12</sub> or cobalamin) is ingested predominantly in meat, fish and dairy products. Stomach acid and intestinal enzymes aid binding of B<sub>12</sub> to intrinsic factor in the duodenum. This intrinsic factor-vitamin complex is then absorbed through the terminal ileal cells by receptor-mediated endocytosis. In the blood 6-20% of cobalamin is bound to holotranscobalamin II (TCII) the physiologically active form of the vitamin (1), the rest to haptocorrins (formerly referred to as TC I or III). The amount of B<sub>12</sub> in the average western diet is 5-15 µg/day, more than sufficient to meet the recommended dietary allowance of 2 µg/day. Therefore, except in strict vegetarians, the presence of cobalamin deficiency implies the presence of an absorptive problem. The majority of cases are caused by pernicious anaemia (lack of intrinsic factor), however atrophic gastritis (common in the older population) can contribute to cobalamin malabsorption (2,3).

Traditionally vitamin B<sub>12</sub> deficiency was thought of as a haematological disorder, where the classical abnormalities of florid pernicious anaemia (anaemia, macrocytosis, low B<sub>12</sub>, glossy tongue, hyper segmented neutrophils and megaloblastic changes in the bone marrow) were easily identified. An abnormal Schilling test would add weight to a diagnosis that poor B<sub>12</sub> absorption was causing the

deficiency. However, it is now well recognised that cobalamin deficiency can present as a neurological or neuropsychiatric disease without concomitant haematological signs (4). Symptoms, such as loss of position and vibration sense, paresthesia, impaired touch or pain perception, ataxia, abnormal gait, and psychiatric disorders, may be present and it is important they are recognised early enough for treatment to be effective.

Measurement of serum vitamin B<sub>12</sub> itself is not a sensitive or specific test for determining deficiency. A low serum B<sub>12</sub> level does not always indicate deficiency (5), and a low normal serum B<sub>12</sub> level does not exclude deficiency (6). This is because of the various forms of B<sub>12</sub> in serum as mentioned above. TCII (the active form) can be measured with a commercial test-kit. However, more research is required to evaluate it for routine purposes.

In humans B<sub>12</sub> is required as a cofactor for two enzymes: methionine synthase (converts homocysteine to methionine) and methylmalonyl CoA mutase (converts methylmalonyl CoA, to succinyl CoA). When B<sub>12</sub> concentrations are sub-optimal, enzyme activities slow down resulting in elevated plasma methylmalonic acid (hydrolysed methylmalonyl CoA) and homocysteine. These metabolite tests are thought to show functional evidence for B<sub>12</sub> deficiency in the tissues (or folate deficiency in the case of homocysteine) and have been used extensively over the past decade or more. The problem is there is no gold standard for diagnosing B<sub>12</sub> deficiency. Some authors consider the metabolic detection method (MMA and homocysteine) to be more sensitive than serum vitamin B<sub>12</sub> (5,7), others do not (8,9). MMA is not specific for B<sub>12</sub> deficiency; renal impairment is the principal cause of false-positive increase (10,11), and it is also raised in thyroid conditions (12) and intravascular volume depletion (13). A cautious approach is to use B<sub>12</sub> as a first line test and to recommend MMA (and homocysteine) as supplementary tests when B<sub>12</sub> is low or low normal (14,15).

The aim of this study was to evaluate a method for serum MMA, determine our own reference range and compare results with TCII and vitamin B<sub>12</sub>. MMA, vitamin B<sub>12</sub>, TCII and creatinine were assayed in serum from 158 "normal" adult volunteers selected randomly from the electoral roll. Data is also presented from 189 patient samples requested for B<sub>12</sub> and/or MMA (52% were 65 years or older, 80% with B<sub>12</sub> levels less than 200 pmol/L. TCII was assayed in 67 of these samples.

## Materials and methods

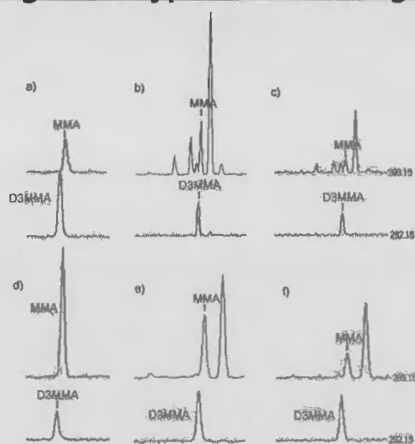
### Methylmalonic acid

The method is a typical stable isotope internal standard gas chromatograph/mass spectrometry (GCMS) technique. The internal standard (D3MMA) is identical to MMA except the three hydrogens on the methyl group have been replaced by deuterium. MMA was purchased from Sigma (Missouri, USA) and ICN Biochemicals (Ohio, USA). D3MMA from Cambridge Isotope Laboratories (Massachusetts, USA). The derivatising reagent n-(tert-butyl)dimethylsilyl-n-methyltrifluoroacetamide (MTBSTFA) is also from Sigma.

Excess sodium chloride and internal standard (50 µL 5 µmol/L) is added to 10 mL screw-topped glass tubes. Patient sample, standard (0.25, 0.5, 1.0 and 2.0 µmol/L) or control (500 µL), and finally salt-



**Figure 1. Typical chromatograms**



Chromatograms using selected ion monitoring for m/z ions 289 and 292.

x-axis: time. Currently MMA elutes at 37.0 minutes and D3MMA at 36.9 minutes.

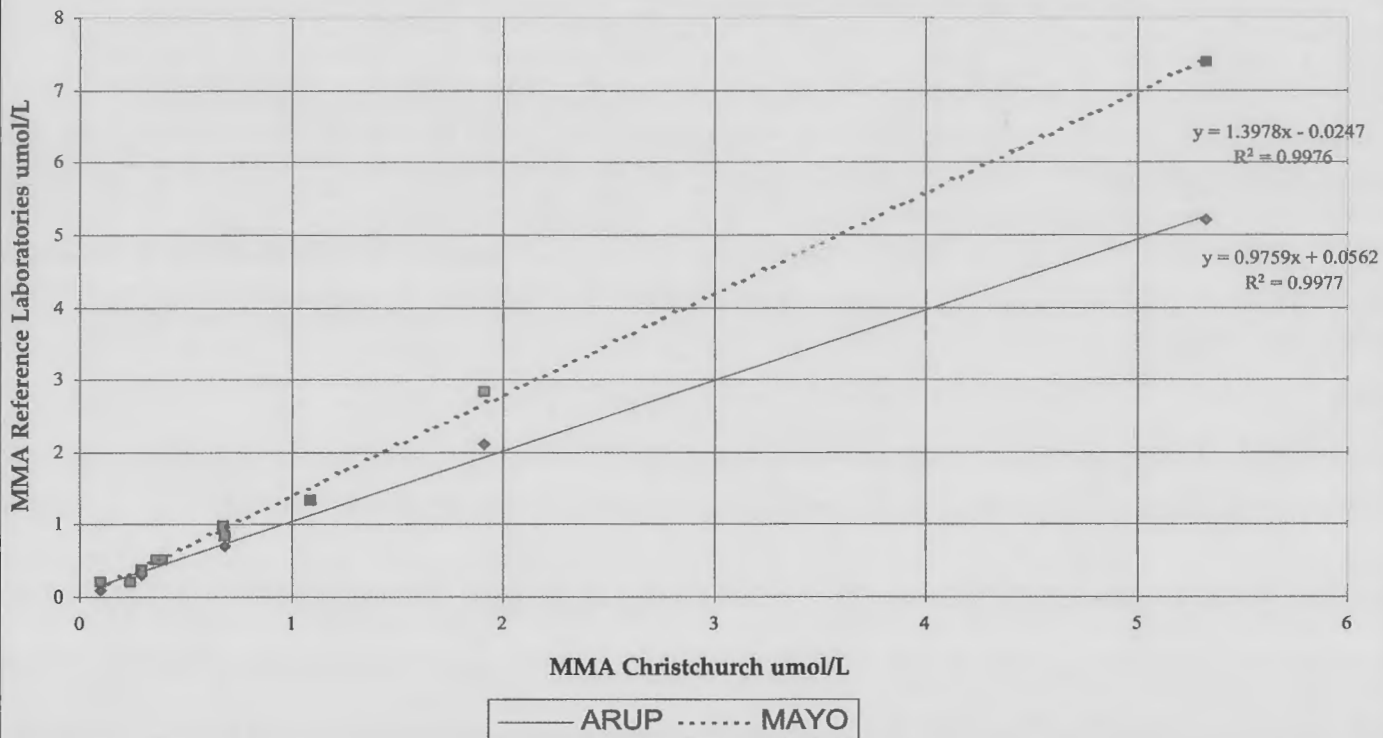
b) and c) time scale is compressed. y-axis: ion abundance.

Top row: current solvent extraction method. Bottom row alternative column extraction method.

In all cases D3 MMA is 0.5 µmol/L

- a) 0.25 µmol/L MMA
- b) e) high serum
- c) f) normal serum
- d) 2.0 µmol/L MMA

**FIGURE 2: MMA extra-laboratory comparison**



saturated hydrochloric acid (500  $\mu\text{L}$  3 mol/L) is added while vortex mixing. The tubes are twice extracted with ethyl acetate (4 mL) followed by centrifugation. Extracts are pooled, a drop of benzene added, then dried under vacuum at 50°C. Benzene aids removal of any water extracted, which interferes with derivatisation. Dried tubes can be capped and stored frozen at this point.

Just before injection, analytes were dissolved well in acetonitrile (50  $\mu\text{L}$ ), then derivatised with excess MTBSTFA (50  $\mu\text{L}$ ) for 60 minutes at 60°C. During derivatisation, sample extracts must be heated under nitrogen to prevent loss of MMA due to oxidation with headspace air to form pyruvate and 2-hydroxy-2-methylmalonic acid derivatives (16).

A Shimadzu QP5000 GC-17A GCMS with a DB-MS5 capillary column: length 30 m, ID 0.25 mm, film thickness 0.25  $\mu\text{m}$  was used to separate and detect the analytes. A DB5 column was recommended as it separates MMA from 3-hydroxyisovalerate, a possible interferent (also produces a 289 ion). Sample (1  $\mu\text{L}$ ) is injected (splitless) onto column with initial flow of helium at 1 mL/minute. The GC injection port temperature is 240°C with an initial oven temperature at 80°C. The oven temperature increases at 2°C per minute from 80 to 137°C and then ramps quickly to 280°C, where it is held for a further 5 minutes. A total run time of 50 minutes is required to separate otherwise co-eluting compounds.

The ions formed in the mass spectrometer are detected by selectively monitoring masses 289.13 and 292.15. These ions are formed after loss of a tertiary butyl group following ionisation of the parent ion. Selected ion monitoring (SIM) increases sensitivity over scan mode by around 3 orders of magnitude. Calibration is achieved by plotting the relative response of ions formed from the MMA standard to those formed from the D3MMA internal standard (peak area 289.13/ peak area 292.15) versus concentration of MMA in  $\mu\text{mol/L}$ .

An extra-laboratory comparison between three laboratories was done: Canterbury Health Laboratories in Christchurch New Zealand; Mayo Clinic in Minnesota, USA (10 samples); and Associated Regional and University Pathologists Inc. (ARUP) in Salt Lake City Utah, USA (5 of the above 10 samples).

It is known that solvent extraction also extracts several nonacidic compounds of similar structure or polarity as MMA (17). A comparison method was set up to compare column extraction clean-up versus solvent extraction (18). CHEM-ELUT columns were purchased through Phenomenex (California, USA) and conditioned by placing on a vacuum manifold and washing sequentially with hydrochloric acid saturated with sodium chloride (2 mL 3 mol/L), de-ionised water (10 mL), methanol (5 mL), and ethylacetate (5 mL). Columns were then dried for approximately 30 minutes.

Again, internal standard (50  $\mu\text{L}$  5  $\mu\text{mol/L}$ ) was mixed with standard MMA, QC or sample (500  $\mu\text{L}$ ), and hydrochloric acid (500  $\mu\text{L}$  3 mol/L), then all the sample applied to the conditioned column. Each column was extracted with ethyl acetate (12 mL), the eluate dried, derivatised and injected into the GCMS as for the solvent extraction method.

### Holotranscobalamin vitamin B<sub>12</sub>

Holotranscobalamin II is the physiologically active metabolite of B<sub>12</sub>, and B<sub>12</sub> is only taken up into the cells in this form. Axis Shield (Oslo, Norway) have recently released a radioimmunoassay test kit using cobalt 57 labeled TCII. The method involves capture of the TCII bound B<sub>12</sub> from samples using magnetic micro spheres coated with monoclonal antibody. These can be separated from the solution using a magnetic separator, washed and measured by an adaptation of the radioimmunoassay ICN vitamin B<sub>12</sub>/folate SNB assay. There is an inverse relationship between concentration and radioactivity measured in the tube. The manufacturers recommend assaying samples in duplicate, but because of cost we processed standards in duplicate but samples and controls singly.

### Vitamin B<sub>12</sub>

Vitamin B<sub>12</sub> was measured using a competitive binding fluorescence immunoassay on the Ciba Corning ACS 180.

### Reference range study

Written informed consent was obtained from volunteers randomly selected from the electoral roll and ethical approval given by the Canterbury Ethics Committee. The distribution by age and gender is shown in Table 1. Samples had been stored at -30°C for 3 years unthawed. According to manufacturers' information, TCII samples are stable kept frozen for up to 10 years. The same is thought to be true for B<sub>12</sub>. MMA is stable despite being repeatedly thawed and refrozen (19). Plasma creatinine was measured on an Abbott Aeroset and creatinine clearance calculated using age and lean body weight.

### Statistical analysis

Subjects assigned as being B<sub>12</sub> deficient (high MMA and low TCII) and those with a substantial decrease in creatinine clearance (less than 1.0 mL/s) were excluded. Excel was used to calculate mean and standard deviation for MMA and TCII in the age and gender groups. A regression equation was calculated using Sigmaplot for correlation of MMA and age. A receiver operating characteristic (ROC) curve was calculated using MedCalc (disease as determined by both abnormal MMA and TCII in all reference range subjects and patients with TCII analysed). The ROC curve was used to determine the optimal upper limit for B<sub>12</sub>, below which, samples should be referred on for MMA analysis. All other analyses were done using SigmaStat on log transformed data for MMA and TCII. For MMA a Mann-Whitney U-test was performed to compare male and female groups, then the overall 97.5% cutoff level determined after back transformation. For TCII a t-test was used to compare gender and calculate power. All tests performed at a significance level of 0.05.

## Results

### Separation

Figure 1 shows typical chromatograms for standards and plasma samples. With standards the 289 peak (top channel of each pair) is MMA, and the 292 peak (bottom channel) is D3MMA. In all chromatograms D3MMA is 0.5  $\mu\text{mol/L}$ . In a) MMA is 0.25  $\mu\text{mol/L}$  i.e. half the D3MMA; and in d) 2.0  $\mu\text{mol/L}$  i.e. four times the D3MMA. D3MMA elutes 0.1 minutes earlier than MMA as a carbon-deuterium bond is slightly shorter than a carbon-hydrogen bond, thus there is less interaction with the stationary phase, and D3MMA is slightly more volatile.

Chromatograms of serum samples extracted with solvents (method being evaluated: b) from an abnormal serum; and c) from normal serum. MMA is just separating from other contaminant peaks eluting before and after. A very slow temperature ramp (2°C/min from 80°C to 138°C) was required to separate these unknown contaminants.

The bottom set of chromatograms is from the alternative sample preparation method using CHEM-ELUT column extraction. These chromatograms are much cleaner, yet as shown in Table 2 both methods give similar results (one plasma sample spiked). Although this alternative method is not currently able to be automated in our laboratory for routine use, it is mentioned here to validate the solvent-extraction method.

### Calibration

The same results were obtained when assays were calibrated with MMA purchased from either Sigma or ICN Biochemicals (normal sample 0.19  $\mu\text{mol/L}$ ; elevated sample 0.70  $\mu\text{mol/L}$ ). MMA and internal

**Table 1. Age and gender distribution for MMA and TCII reference range subjects, with mean and standard deviation for respective groups.**

Age in years	total number	Females		total number	Males	
		MMA mean $\pm$ sd	TCII mean $\pm$ sd		MMA mean $\pm$ sd	TCII mean $\pm$ sd
<35	19	0.173 $\pm$ 0.063	46.8 $\pm$ 17	20	0.173 $\pm$ 0.057*d	64.2 $\pm$ 23*i
35 -<50	26	0.189 $\pm$ 0.077*a	60.0 $\pm$ 33*g	16	0.176 $\pm$ 0.087	71.6 $\pm$ 37
50 -<65	27	0.197 $\pm$ 0.063*b	67.5 $\pm$ 32*h	24	0.198 $\pm$ 0.098*e	64.0 $\pm$ 41*j
65 -<84	13	0.187 $\pm$ 0.012*c	67.1 $\pm$ 31	13	0.226 $\pm$ 0.062*f	60.5 $\pm$ 27
Total	85	0.187 $\pm$ 0.067	60.4 $\pm$ 30	73	0.189 $\pm$ 0.081	65.1 $\pm$ 33

\*The number of subjects excluded from each group because either they were clearly B12 deficient or had creatinine clearance <1.0 mL/s a=2, b=3, c=10, d=1, e=1, f=4, g=2, h=2, i=1, j=1.

**Table 2. Comparison of solvent extraction and column extraction methods for serum**

serum	Solvent extraction method being evaluated	Alternative Chem-elut column extraction
Q1	0.39 $\mu$ mol/L	0.41 $\mu$ mol/L
Q2	0.69 $\mu$ mol/L	0.62 $\mu$ mol/L
Q3	5.33 $\mu$ mol/L	6.8 $\mu$ mol/L

**Table 3. Calibration comparison with and without human serum albumin**

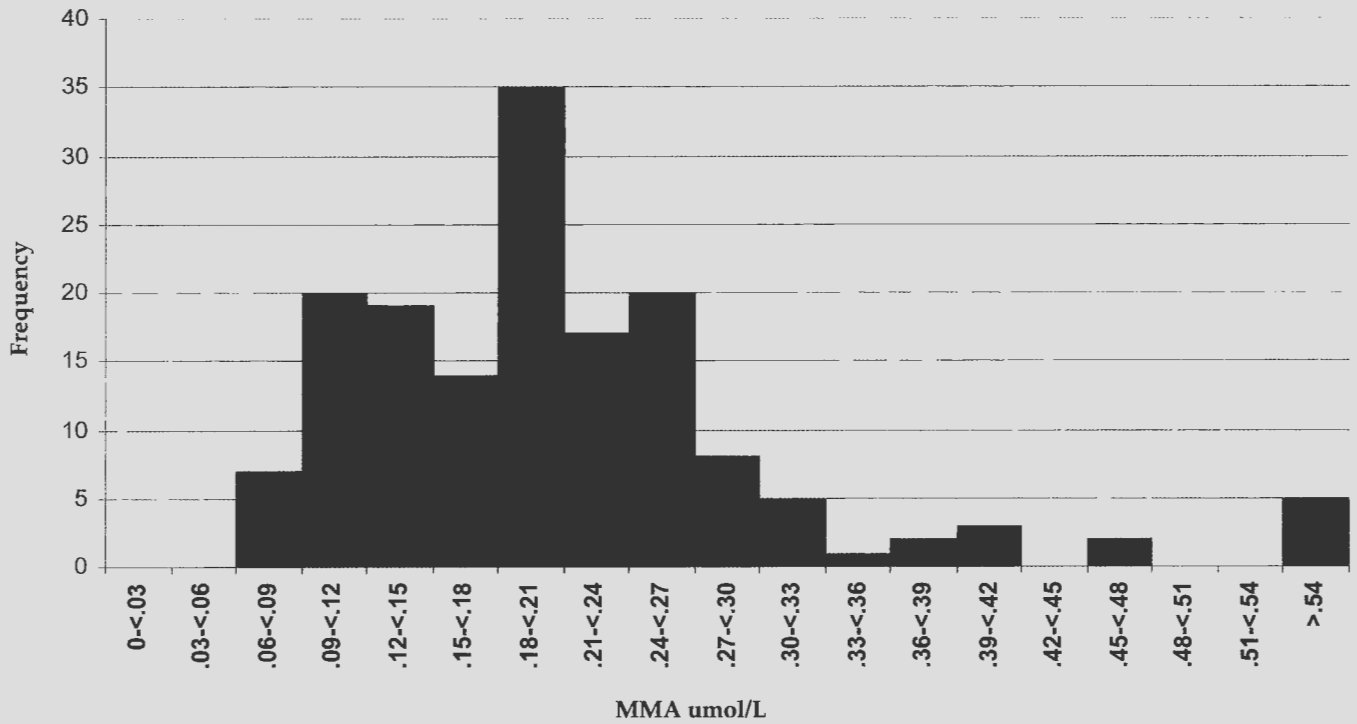
sample	standards without protein		standards with protein	
	289/292	concentration $\mu$ mol/L	289/292	concentration $\mu$ mol/L
0.25	0.454		0.453	
0.5	0.99		0.992	
1.0	1.966		1.958	
2.0	3.778		3.737	
normal serum	0.458	0.25	0.458	0.23
QC2	1.122	0.60	1.122	0.58

**Table 4. Extraction efficiency for MMA and D3MMA in ethyl acetate**

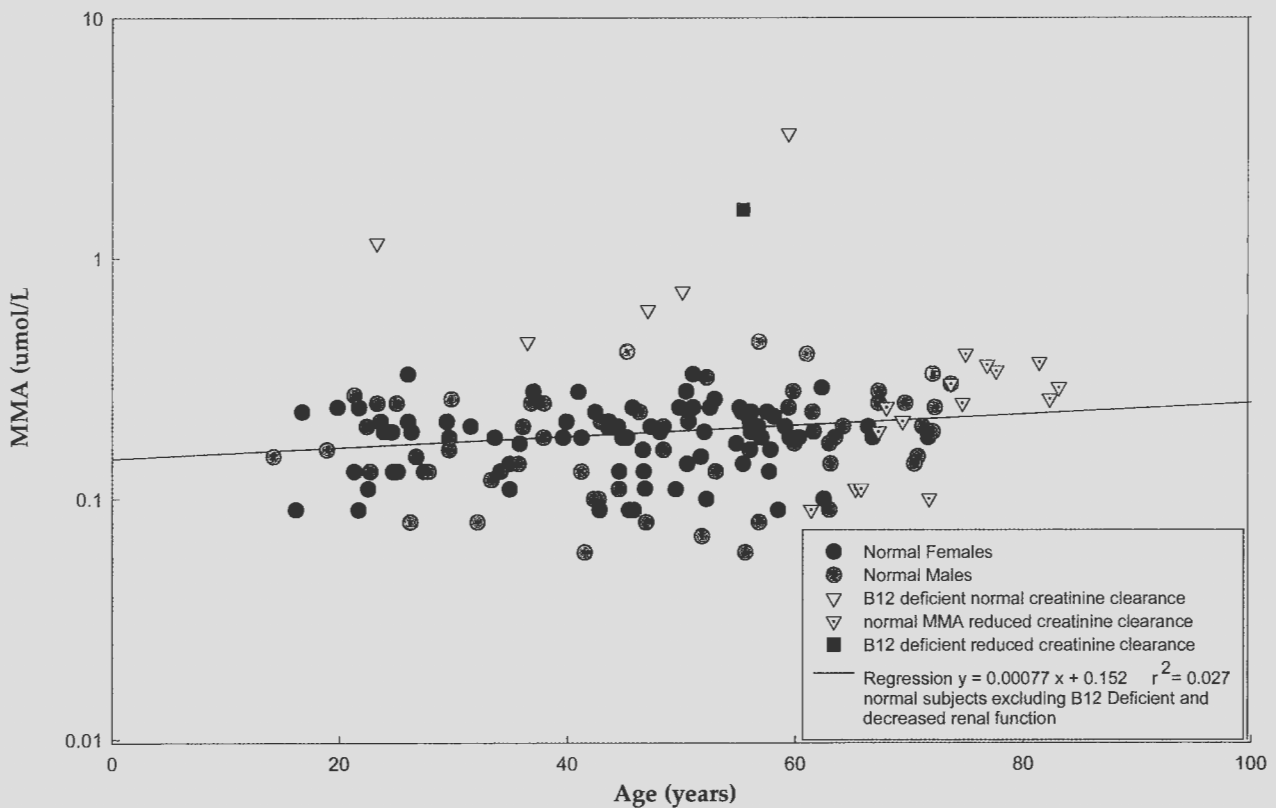
Extraction with ethylacetate	Cumulative extraction efficiency			
	MMA peak area	% MMA	D3MMA peak area	% D3MMA
first 4 mL	46329	76%	13699	78%
second 4 mL	58732	96%	17168	98%
third 4 mL	61315	100%	17495	100%



**FIGURE 3: Frequency histogram for MMA reference range subjects**



**FIGURE 4: MMA versus Age in all Reference Range Subjets**



**Table 5. Recovery of spiked MMA in serum**

Amount of MMA added $\mu\text{mol/L}$	Batch A		Batch B	
	concentration $\mu\text{mol/L}$	Recovery	concentration $\mu\text{mol/L}$	Recovery Batch B
0	0.09		0.11	
0.3	0.38	97%	0.40	97%
0.6	0.67	97%	0.70	98%
6.0	5.25	85%	5.40	88%

**Table 6. Result summary for external QC programme (Australian Society of Inborn Errors of Metabolism)**

Sample:	Added MMA $\mu\text{mol/L}$	Measured MMA $\mu\text{mol/L}$											
		Lab 1 A-D	Lab 1 E-H	Lab 2 A-D	Lab 2 E-H	Lab 3 A-D	Lab 3 E-H	Chch A-D	Chch E-H	Lab 5 A-D	Lab 5 E-H	Lab 6 A-D	Lab 6 E-H
A/E	0	0.6	0.6	0.47	0.32	0.26	0.50	0.14	0.19	<5	<5	0.5	No
B/F	0.62	1.1	1.3	1.99	1.83	1.10	1.29	0.76	0.76	<5	<5	1.4	
C/G	2.47	3.1	3.2	6.5	3.33	2.50	3.79	2.46	2.68	<5	<5	3.1	
D/H	10.00	10.3	11.5	22.3	10.84	9.3	12.07	9.3	10.3	8	9	11	Results
Endogenous MMA,calculated		0.61	0.53	NC	0.76	0.41	0.57	0.19	0.16	NC	NC	0.57	NC

NC = not calculated

**Table 7. Sample stability at room temperature and 4°C**

number of days stored	Sample A at room temperature $\mu\text{mol/L}$	Sample B at 4°C $\mu\text{mol/L}$
1	0.89	0.74
2	0.79	0.74
4	0.90	0.65
6	0.95	0.74
9	0.85	0.70
15	0.87	0.70
30		0.80



standard run through the procedure without the other gave only the one expected peak (i.e. there was no D3MMA in the MMA, and no MMA in the D3MMA).

#### Aqueous calibration versus standards in human serum albumin

The same results were obtained when standards contained human serum albumin (50 g/L) added before acidifying and extraction. The extraction efficiency of standards in an aqueous or protein containing matrix is the same, i.e. no matrix effect. (Table 3).

#### Extraction efficiency

To compare rates of extraction of MMA and internal standard each of 3 successive 4 mL solvent extracts of a high MMA serum sample (1.8 µmol/L) was derivatised. The extraction of both compounds was virtually identical (Table 4).

#### Linearity

The method is linear up to 6 µmol/L giving a line of best fit  $y = 0.97x + 0.08$ ,  $r^2 = 0.997$  where  $y$  is the measured concentration of MMA, and  $x$  the actual concentration of MMA.

#### Recovery

Recovery is 97-98% for normal and slightly elevated levels of MMA (Table 5), and although our own earlier results showed a recovery of 85% and 88% at 5.3 µmol/L, our external QC results (Table 6) showed good recovery right across the range (and 93% and 101% at a level of 10 µmol/L).

#### Stability

A stability study was undertaken using two different samples: one left at 15-20°C on a sunny bench; the other in the refrigerator at 4°C. They were stable at least 15 days at room temperature, and at least a month refrigerated (Table 7).

#### Interferences

**Anticoagulant:** Serum, heparinised or EDTA plasma from one individual all gave the same results (0.16, 0.17, 0.17 µmol/L respectively).

**Haemolysis:** A low and high sample were diluted slightly with either distilled water or the same volume of saline washed red cells lysed with distilled water (final haemoglobin concentration 5 g/L). The MMA results in water-diluted versus haemolysis-diluted samples were: for a low sample 0.33 and 0.28 µmol/L; and for a high sample 0.85 versus 0.91 µmol/L, respectively.

#### Between batch precision

Over 44 batches, the coefficient of variation at 0.30 µmol/L was 5.3%, and at 0.70 µmol/L was 4.5%.

#### Limit of detection

A known high control sample was diluted in MMA-free human serum albumin. Samples with theoretical values of: 0.163, 0.108, 0.081, 0.065 and 0.054 µmol/L gave measured values of: 0.17, 0.12, 0.08, 0.04, 0.03 µmol/L respectively. Although this experiment was only assayed once, the effective limit of detection was 0.08 µmol/L.

#### Extra-laboratory comparison for accuracy

Samples were analysed in Christchurch and sent to two laboratories in the United States. All laboratories correlated well with  $r^2 = 0.998$ ; the Mayo Institute's results were 40% higher than those from Christchurch and ARUP laboratories (Figure 2).

## Reference range study

### MMA

The frequency distribution for MMA in these 158 subjects was not normal (Figure 3). Excluded from the normal range data were:

- 6 subjects clearly B<sub>12</sub> deficient (based on high MMA and low TCII)
- 15 subjects with a substantial decrease in creatinine clearance (less than 1.0 mL/s)

In the remaining 137 subjects there was no correlation of MMA ( $y$ ) with age ( $x$ ); equation  $y = 0.0008x + 0.155$ ,  $r^2 = 0.027$ , (Figure 4). Using Mann-Whitney U-test (excluding only B<sub>12</sub> deficient subjects), the difference between male and female groups was not significant ( $P=0.702$ , the power of the performed test was 0.05; at a significance level of 0.05 and is below the desired power of 0.800). Using SigmaStat with log transformed data, 97.5% of the population were below 0.35 µmol/L (mean 0.173, median 0.180, sd 0.171, confidence interval of the mean 0.0289).

Figure 4 shows results of age versus MMA with normal and reduced creatinine clearance (<1.0 mL/s). All subjects, except one male, had a normal plasma creatinine (exception: one male 67 years with creatinine of 0.12 mmol/L, creatinine clearance 0.82 mL/s and MMA 0.19 µmol/L). There were six subjects with MMA greater than 0.35 µmol/L and with a normal TCII. Of this group, both females and one male had a low renal clearance. The remaining three subjects had slight elevations of MMA (0.40, 0.41 and 0.45 µmol/L respectively).

### TCII

The frequency distribution for TCII is shown in Figure 5. The lower 2.5 percent cut-off in our population using log-transformed data was 25 pmol/L (B<sub>12</sub> deficient subjects excluded). The manufacturer quotes a TCII reference range of greater than 27 pmol/L. Student t-test on log transformed data showed no significant statistical difference at a level of 0.05 between all males and all females ( $P=0.188$ ). However, there was a significant difference between females younger than 35 years and men 35-50 years of age ( $P=0.005$ ; power=0.805) see Table 1 and Figure 6. There was a significant difference between females younger than 35 years and women over 50 years of age, but the power was less than the desired power of 0.800 ( $P=0.008$ ; power = 0.729). There were four subjects with TCII less than 27 pmol/L who were not deficient based on MMA. Excluding these four subjects from the reference range data did not alter the calculated MMA cut-off.

### Patient results

Patient results are presented in Figures 7 to 10. One hundred and eighty nine (189) patients were tested for MMA and 67 of these for TCII. The MMA and TCII agreed in 48 patients (72%). Exceptions:

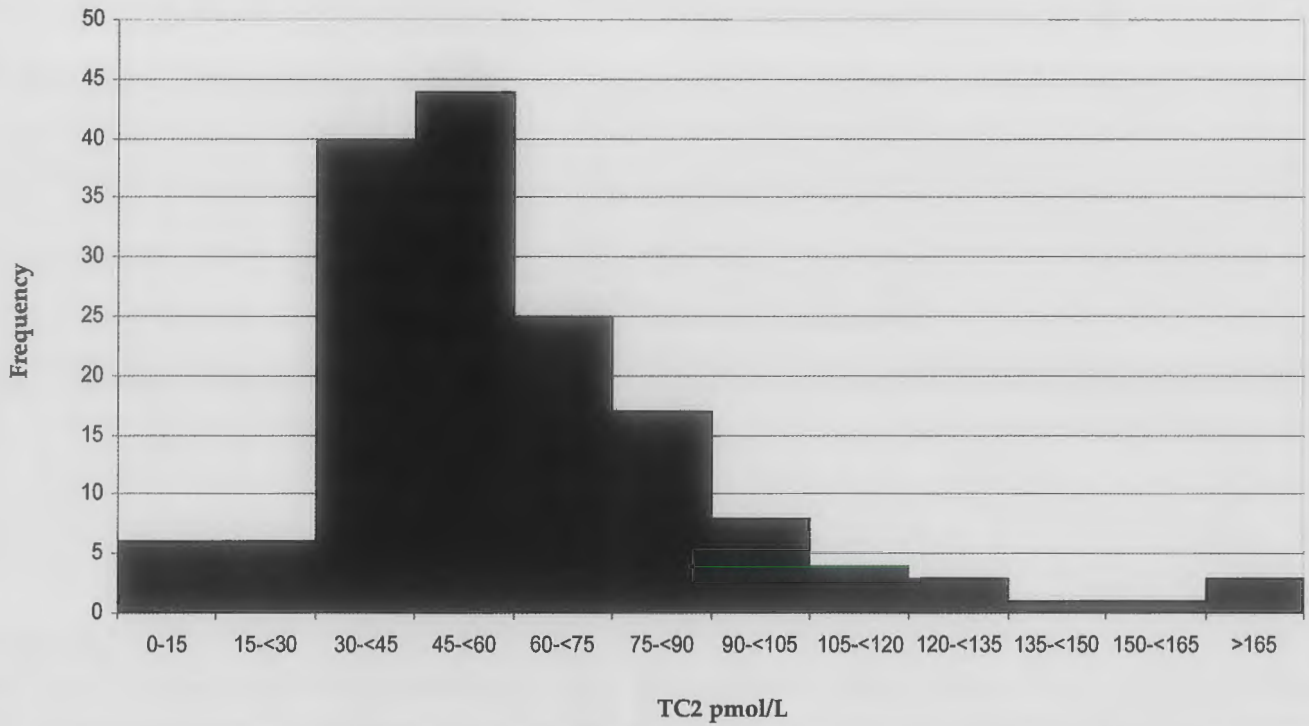
- In nine the MMA was normal with a low TCII.
- In two women (86 and 78 years), MMA was slightly elevated (0.42, 0.47 µmol/L), TCII was at the lower end of normal (29, 36 pmol/L).
- One male (81 years), MMA 0.44 µmol/L, TCII 47 pmol/L, elevated plasma creatinine.
- One patient possibly recently B<sub>12</sub> supplemented (MMA 0.42 µmol/L TCII 320 pmol/L).
- One patient (78 years, female), MMA 0.9, TCII 30 pmol/L, plasma creatinine 0.10 mmol/L.

Out of 21 patients defined as B<sub>12</sub> deficient (high MMA, low TCII), four patients had relatively high vitamin B<sub>12</sub> levels (198, 195, 194, 187 pmol/L). Values for MMA were: 2.77, 1.33, 1.18 and 1.18 µmol/L, and TCII: 21, 110, 110, 110 pmol/L respectively.

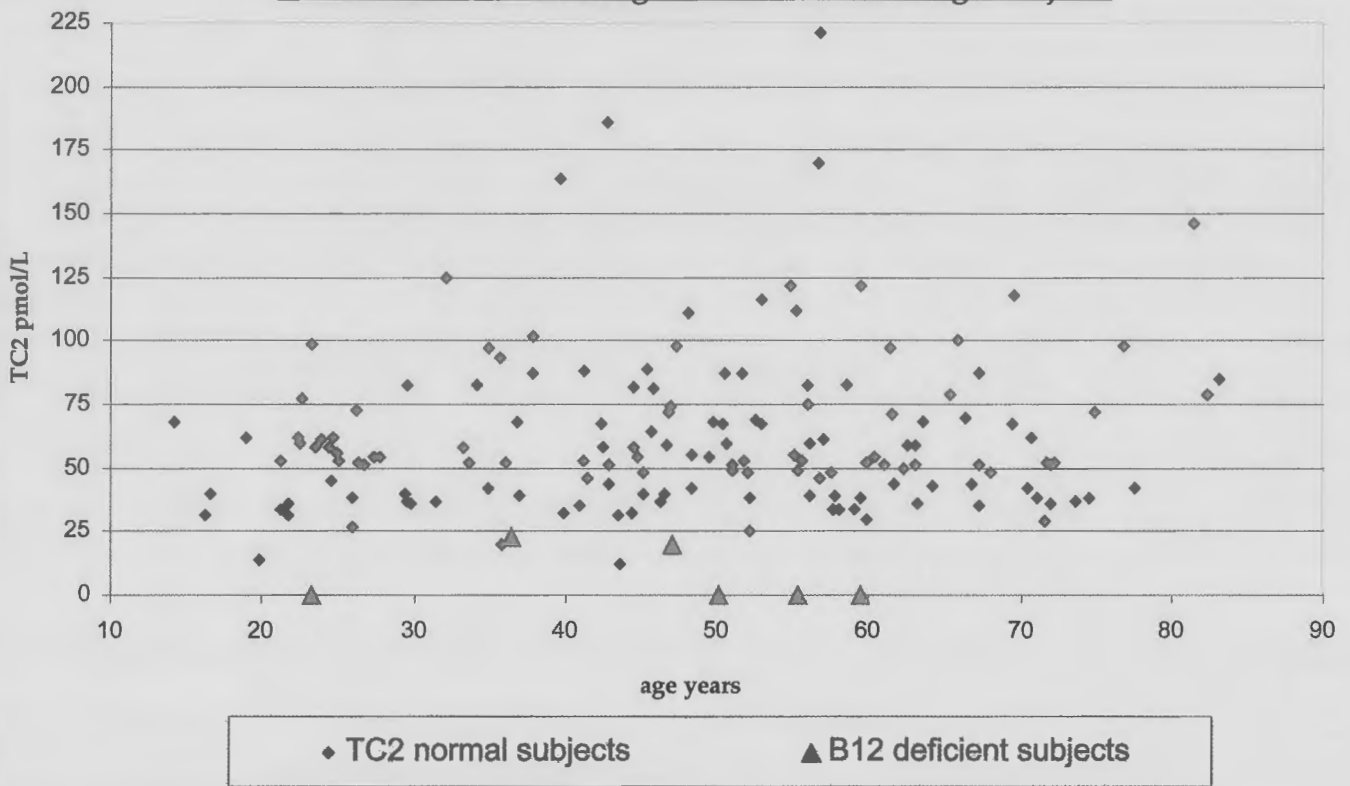
### Reference range subjects and patients

Out of 58 people with B<sub>12</sub> levels between 150 and 200 pmol/L, 14 had an abnormal MMA and 13 (93%) of those had abnormal TCII

**FIGURE 5: Frequency histogram for TC2 reference range subjects**

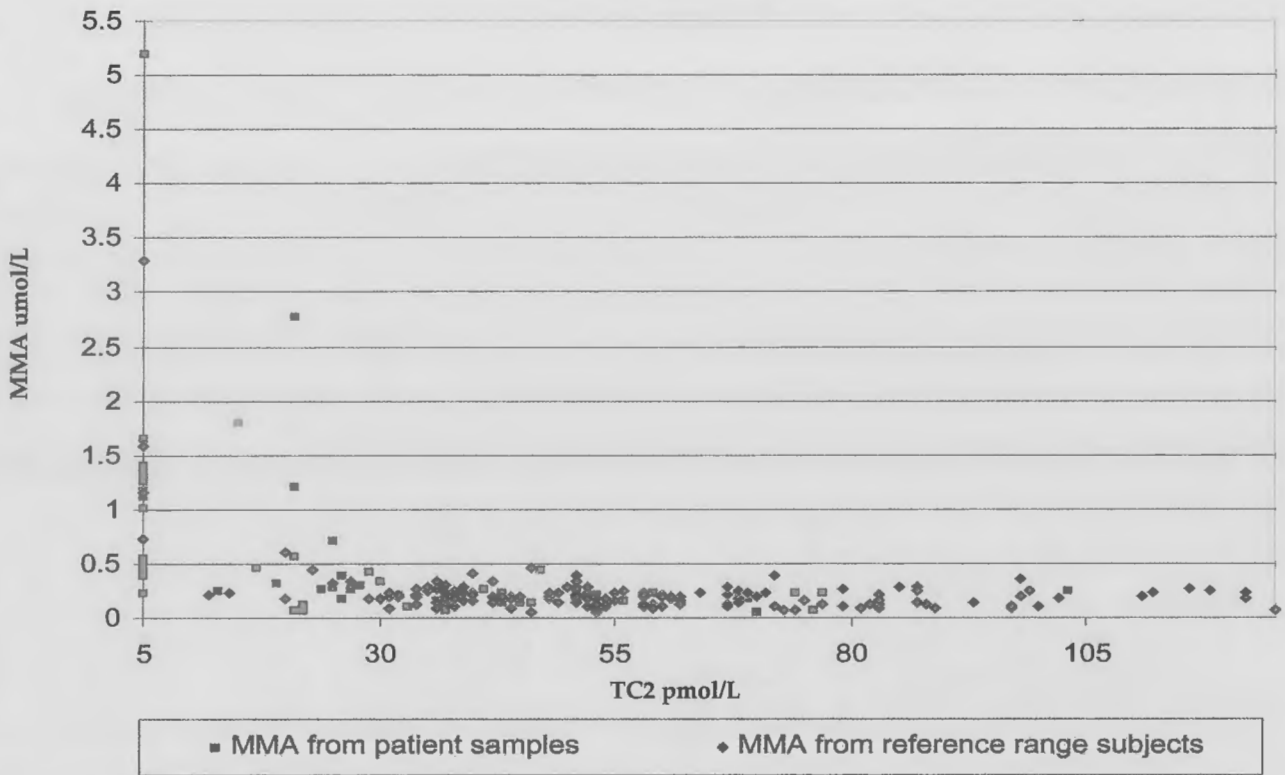


**FIGURE 6: TC2 versus age in all reference range subjects**

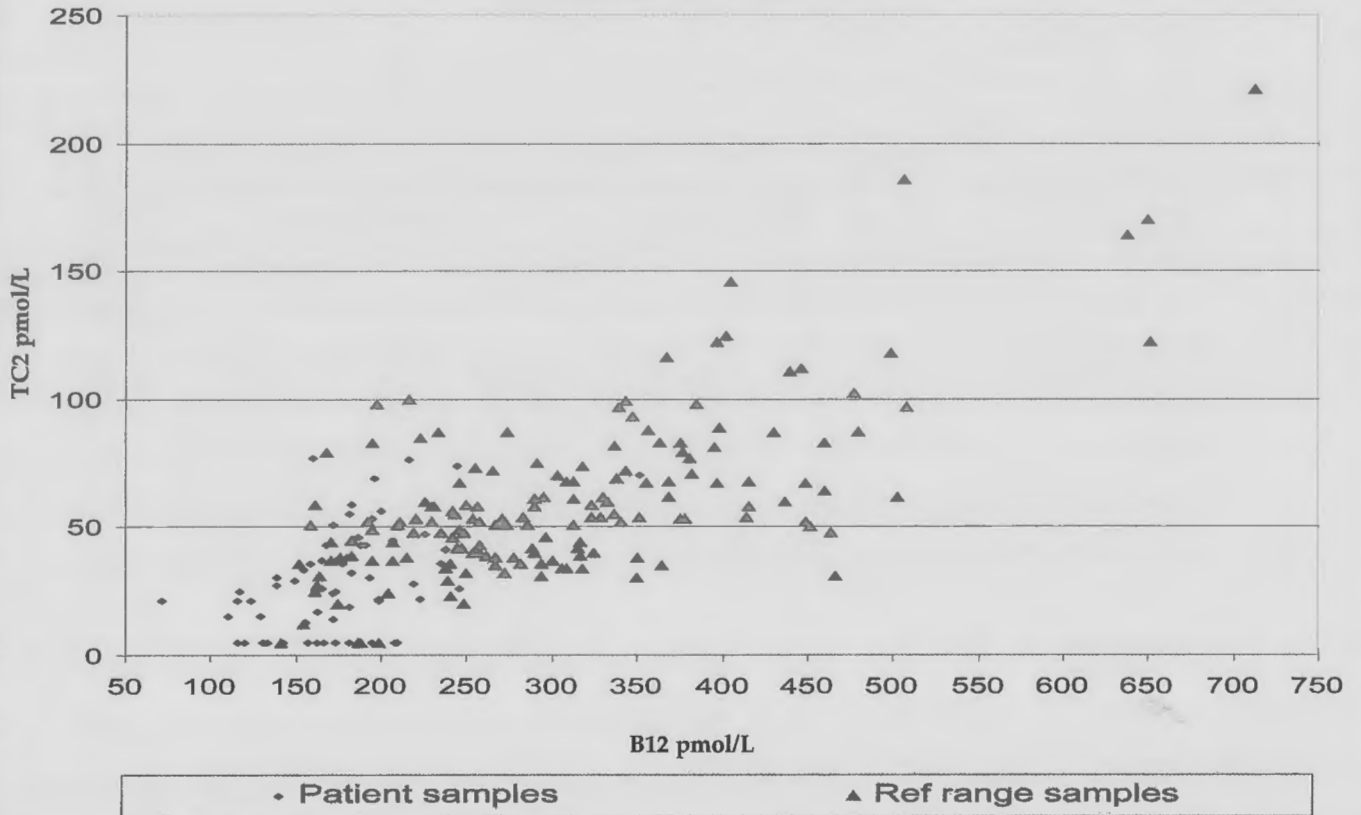




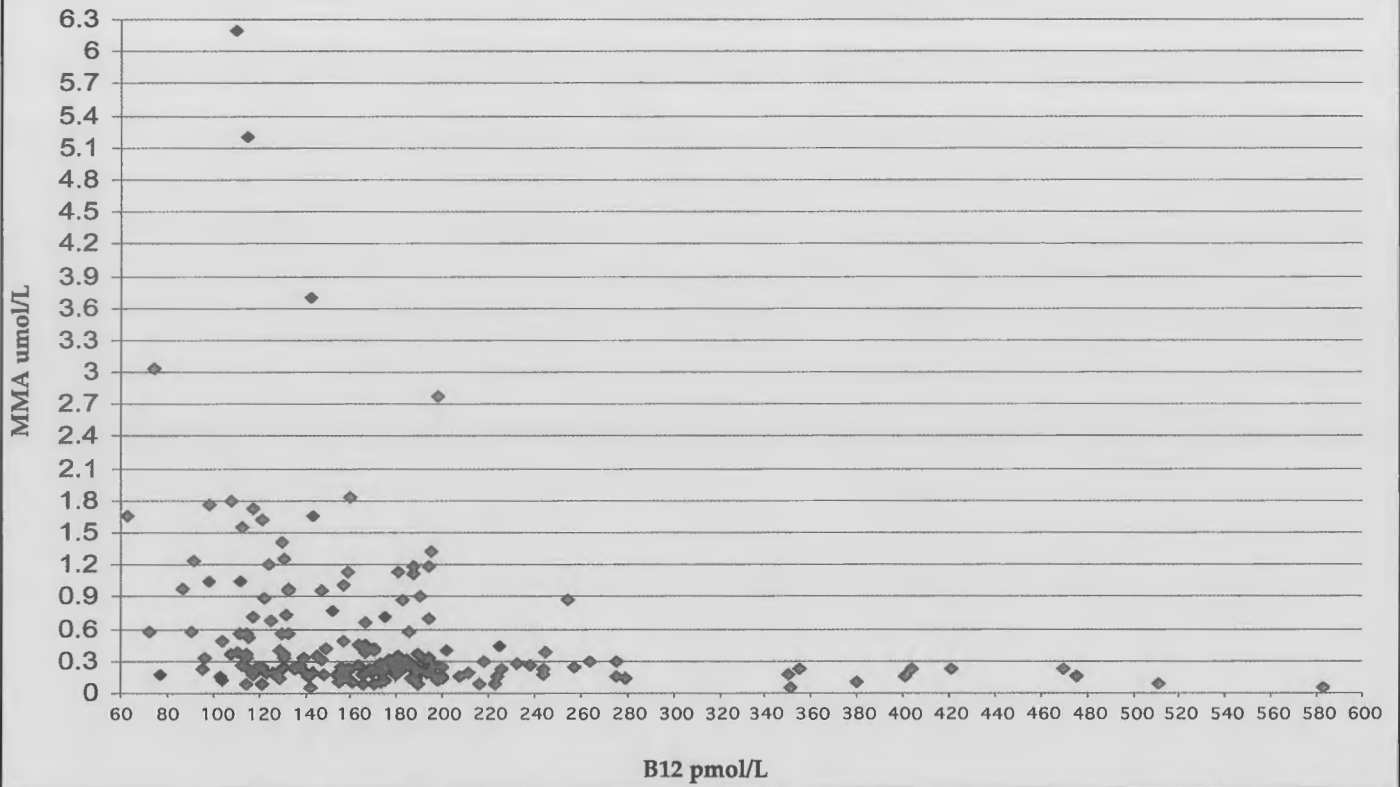
**FIGURE 7: MMA versus TC2**



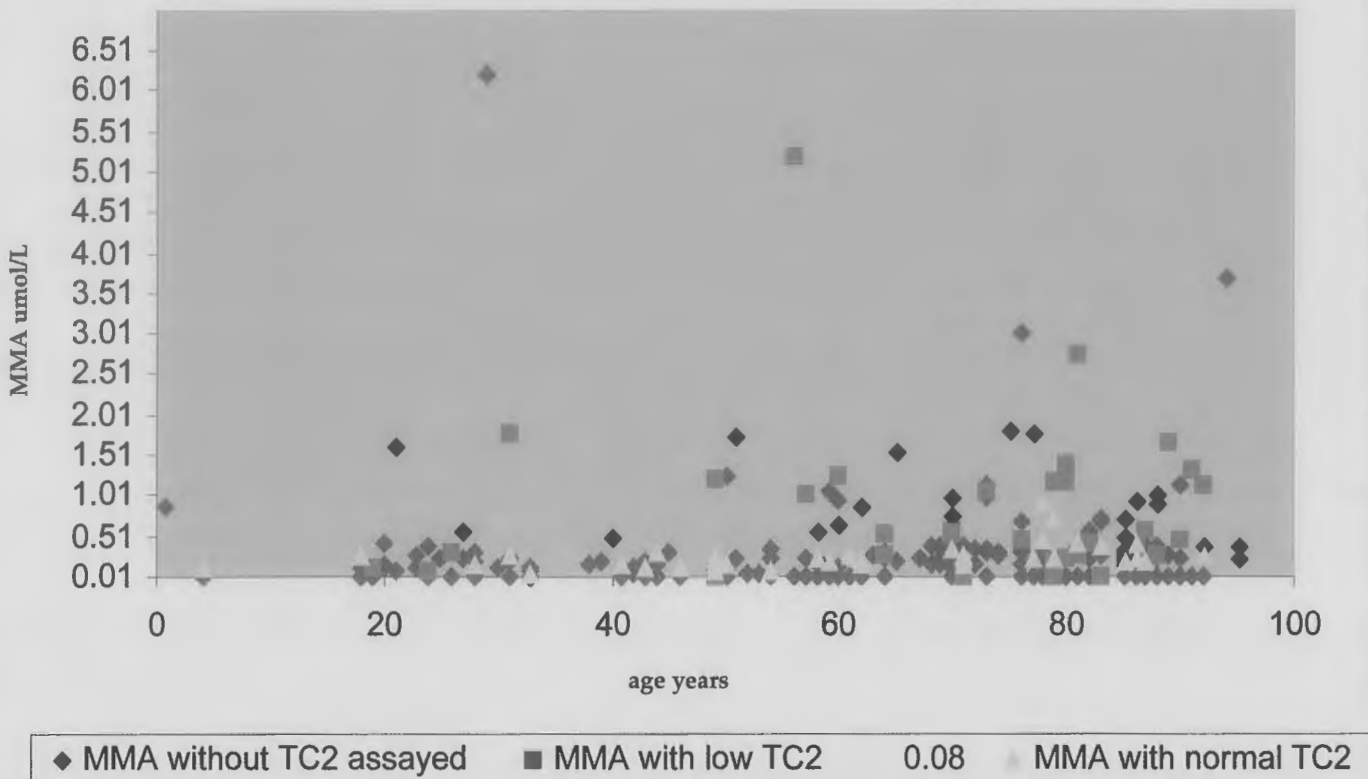
**FIGURE 8: TC2 versus B12**



**FIGURE 9: MMA versus B12 in patients samples**

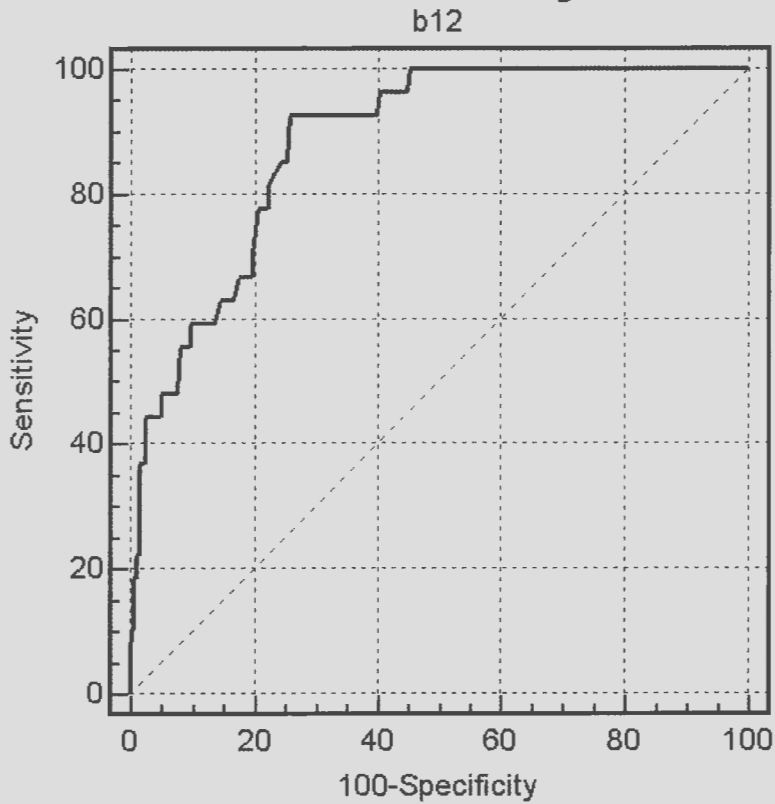


**FIGURE 10: MMA versus age and TC2 in patient samples**



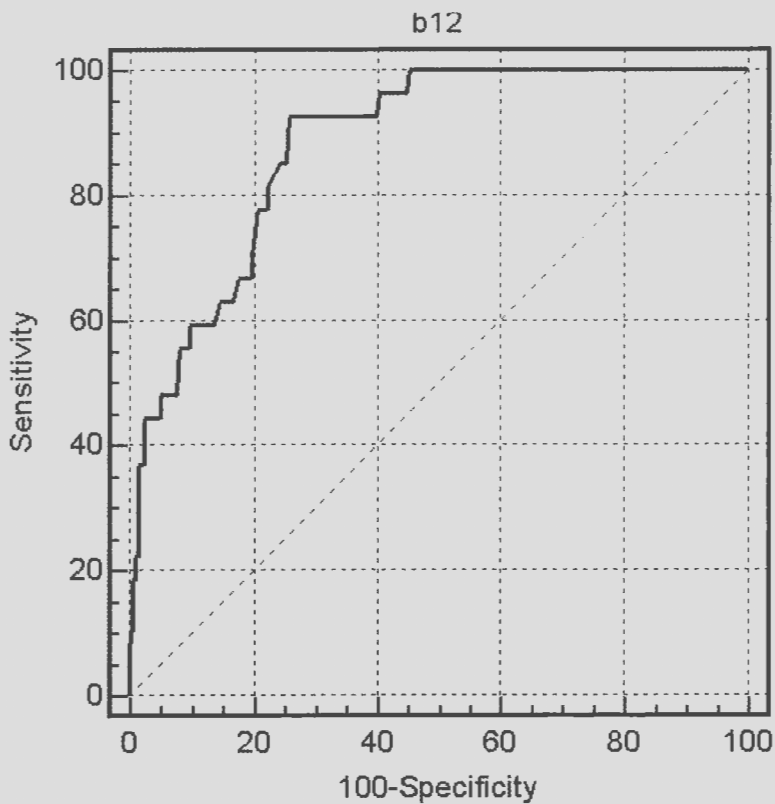


**Figure 11. ROC curve**



A Receiver Operating Characteristic (ROC) curve plotting true positive rate (sensitivity) against false positive rate (specificity) at different cut-off points.

**Figure 12. Vitamin B12 values in subjects with and without B12 deficiency**



Vitamin B12 values in subjects with B12 deficiency (low TCII, high MMA) n+27 and those without B12 deficiency n+198. Solid line indicates optimal cut-off level (198 pmol/L) from ROC curve analysis.

levels, the remaining 44 had a normal MMA, of which 38 (86%) also had normal TCII.

The optimal cut-off for vitamin B<sub>12</sub> using ROC curve analysis was found to be 198 pmol/L (Figure 11 and 12). This cut-off gives a sensitivity of 92.6% (95% confidence interval: 75.7-98.9) and a specificity of 74.2% (95% confidence interval: 67.6-80.2). The positive predictive value was 32.9% (probability that disease is present when the test is positive) and negative predictive value was 98.7% (probability that the disease is not present when the test is negative). Diagnostic accuracy was 88.3 (area under ROC curve) with a standard error of 0.026 and a 95% confidence interval of 0.833 to 0.922.

## Discussion

This study shows that the solvent extraction method was robust and reliable for measurement of MMA and the external quality assurance program results reinforces this view (Table 6). The MMA reference range was found to be in agreement with other published ranges (20), with no significant differences in age or gender. As expected, it was found that as B<sub>12</sub> decreases so does TCII, while MMA increases. Unfortunately the patient data is of limited use because there was no clinical assessment done, that being beyond the purpose of this study. However, MMA and TCII noticeably became abnormal in some subjects when B<sub>12</sub> levels drop below 200 pmol/L.

An international study published in 1999 compared MMA results from 13 laboratories and found there was an urgent need to improve poor precision, and address calibration issues (20). To achieve the best precision possible, 2.5 nmol of D3MMA is used in Christchurch, this being the amount of MMA found in 500 µL of borderline patient sample. Ion abundance for both MMA and internal standard should be at least 50,000 mV. To improve accuracy, standard values of 0.25-2.0 µmol/L are used, thus optimising for low and slightly increased MMA concentrations. Samples above this are repeated in dilution.

The calculated upper limit of the reference range was 0.35 µmol/L. Clinical judgment suggests that a suitable upper limit for patient samples should be 0.4 µmol/L. It was found that neither age nor gender affected the reference range, which agrees with earlier work by Nexo and colleagues (21). In our small group we excluded 15 subjects aged 61 to 83 from the reference range data because the creatinine clearance was less than 1.0 mL/s. The MMA in these 15 subjects was still within our clinically decided reference range (0.4 µmol/L or less).

As expected, there was a trend for MMA to increase as TCII became abnormally low (below 27 pmol/L; Figure 7). There was also considerable inter-individual variability (Figure 10):

- 14 people had normal MMA and low TCII (three with TCII <10 pmol/L), are perhaps yet to display a functional deficiency
- Three females (aged 31, 49 and 81 years) had MMA >1.0 µmol/L with only slightly reduced TCII (15, 21 and 21 pmol/L). B<sub>12</sub> values were: 110, 124 and 198 pmol/L respectively.

Also, as expected, TCII increases along with B<sub>12</sub> (Figure 8) and MMA increases as B<sub>12</sub> decreases (Figure 9). Interestingly, in some adult patients with B<sub>12</sub> levels below 200 pmol/L (80% of our patient population), MMA can start to become noticeably abnormal and TCII levels can drop below 10 pmol/L. Swain suggests serum vitamin B<sub>12</sub> should be used as a screening test, with high levels ruling out vitamin B<sub>12</sub> deficiency, levels between 150-350 pmol/L requiring confirmation with MMA and levels lower than 150 pmol/L probably not needing MMA confirmation (14). Hvas and colleagues suggest MMA be analysed when B<sub>12</sub> is between 125-250 pmol/L (15). Unfortunately no method or reference range was given with these recommendations. From ROC curve analysis (Figure 11 and 12) our results suggest it is prudent to measure MMA in patients suspected of having B<sub>12</sub>

deficiency, especially when vitamin B<sub>12</sub> levels are below 200 pmol/L (ACS 180). This analysis is based on the assumption that low TCII and high MMA will correctly categorize patients, which may or may not be true. Wickramasinghe and Ratnayaka concluded that low TCII was not specific for cobalamin abnormality as determined by the sensitive deoxyuridine suppression test (22), and another study mentioned low TCII in folate deficiency (23).

Caution has been urged in the interpretation of elevated MMA in the absence of other evidence. However, in groups at high risk of developing B<sub>12</sub> deficiency (including people who have had gastrectomy and the elderly), it is reasonable to resort to metabolite assays to resolve discrepancies between the serum B<sub>12</sub> level and the clinical and haematological profile. A normal serum B<sub>12</sub> in a patient suspected of having cobalamin deficiency or a low serum B<sub>12</sub> level without convincing evidence of deficiency certainly justifies measurement of metabolite levels (24). However, further clinical studies are required to determine if B<sub>12</sub> deficiency can be excluded when levels of plasma B<sub>12</sub> are low and the plasma MMA is normal (25).

## Conclusions

The GCMS MMA method was found to be accurate, precise and robust. The reference range was clinically determined to be <0.4 µmol/L. We suggest it is prudent to measure MMA in patients suspected of having B<sub>12</sub> deficiency when vitamin B<sub>12</sub> levels are below 200 pmol/L (ACS 180).

## Acknowledgements

Thanks to NSW Biochemical Genetics Service for their MMA method used for monitoring patients with Methylmalonyl CoA Mutase Deficiency. Endocrinology for obtaining ethical approval and providing reference range samples. Specialist Haematology Canterbury Health Laboratories, Biochemistry MedLab South and Southern Community Laboratories Christchurch for sample collection and B<sub>12</sub> analysis

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# Monoclonal gammopathies: A laboratory perspective

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## Abstract

**Background:** Monoclonal gammopathies result from the over-proliferation of a clone of plasma cells or B-lymphocytes. The disorders associated with monoclonal gammopathies may be classified as "plasma cell dyscrasias", "lymphoproliferative disorders", "protein infiltrative and deposition diseases", and "miscellaneous disorders". Monoclonal gammopathy is most commonly associated with monoclonal gammopathy of undetermined significance, followed by myeloma, Waldenström's macroglobulinaemia, amyloidosis and others.

**Objectives:** To review the various techniques available for the detection and characterisation of serum and urine monoclonal proteins and to examine the advantages and disadvantages of these techniques. To discuss the role of the medical laboratory in regard to screening and diagnosis, patient management, use of other tests, and monitoring treatment.

**Method:** A literature review was performed utilising search engine functions on the internet (world wide web), and papers were obtained from original publications or downloaded from the internet. Seven clinical haematologists were contacted through individual meeting with the author or via e-mail.

**Conclusions:** A review is presented to provide information to allow the medical laboratory a critical understanding of techniques available for detecting and identifying monoclonal proteins. The diagnostic issues that face the clinician are presented to allow the medical laboratory and clinician to work in conjunction to provide the best outcome for the patient.

**Keywords:** Monoclonal gammopathies, monoclonal peak, serum electrophoresis, immunofixation.

## Introduction

Monoclonal gammopathies result from an over-production of immunoglobulin from a single clone of plasma cells or B-lymphocytes. The monoclonal protein is recognised as a band of restricted migration on serum or urine electrophoresis. The abnormal cells may produce an intact immunoglobulin, light chains without heavy chains, or rarely heavy chains only (1). In 1937, Tiselius described the separation of serum globulins into  $\alpha$ ,  $\beta$  and  $\gamma$  regions, although the concept of immunoglobulins was not proposed until the late 1950s (2). Now, monoclonal proteins are routinely detected in the modern medical laboratory, and once sophisticated techniques are now used daily to characterise these bands. The detection, quantitation, and monitoring of monoclonal proteins and the rationale behind such testing has been widely discussed in medical literature and will be summarised in this paper.

## Disorders associated with monoclonal gammopathies

The disorders which are associated with monoclonal gammopathies may be classified as "plasma cell dyscrasias", "lymphoproliferative disorders", "protein infiltrative and deposition diseases", and "miscellaneous disorders".

## Plasma cell dyscrasias

### Plasma cell myeloma (PCM, Myeloma)

This is a neoplastic disease of B-cell lineage resulting in the proliferation of a clone of plasma cells (3). Approximately 80% of myeloma cases have a heavy chain with a corresponding light chain. In light chain myeloma (5-10% of myeloma cases), the plasma cell produces only light chains, while in patients with non-secretory myeloma no heavy or light chains are detected because of either failure of production or blocking of secretion (4). Symptomatic patients often present with the classical "diagnostic triad" (5): the bone marrow usually demonstrates >10% bone marrow plasma cells (BMPC); the serum or urine contains a monoclonal protein in 95% of patients; and lytic bone lesions are found in 75% of patients (6). Significant clinical features are bone pain and pathologic fractures due to increased osteoclastic bone reabsorption; fatigue caused by anaemia; nausea, confusion and polyuria due to hypercalcaemia; nausea and fatigue from severe renal failure; and recurrent infections due to multiple factors including marked depression of uninvolved immunoglobulins (6).

### Smoldering myeloma

Approximately 20% of newly referred cases present with asymptomatic myeloma. It is characterised by the presence of >10% BMPC and a monoclonal protein >25 g/L. Patients may remain stable for many years, although eventually most will require treatment for myeloma (7).

### Solitary plasmacytoma

The diagnosis depends on histological proof that the tumour consists of plasma cells identical to those in myeloma and that no other evidence of myeloma exists (4). Approximately 50% of patients have a low-level monoclonal protein in their serum or urine, but uninvolved immunoglobulins are not suppressed. Tumours may be in the bone or extramedullary; most frequently involving sinuses and nasopharynx (6).

### Osteosclerotic myeloma (POEMS syndrome)

This is characterised by peripheral neuropathy, organomegaly, endocrine deficiency, monoclonal protein and skin pigmentation (6). IgA is the most common heavy chain immunoglobulin and 90% of patients have lambda light chain. The monoclonal protein is usually small and the bone marrow is rarely diagnostic, therefore diagnosis is based on the finding of monoclonal plasma cells in an osteosclerotic lesion (8).

## Lymphoproliferative disorders

### Waldenström's macroglobulinaemia (WM)

This describes the clinical condition of patients with a B-cell lymphoma that secretes large amounts of monoclonal IgM (9). Clinical features are usually related to tumour infiltration and to the amount and specific properties of monoclonal IgM (10). Patients may present with lymphadenopathy, splenomegaly, and marrow infiltration to a degree that causes severe anaemia. Complications associated with the elevated concentration of IgM, may include hyperviscosity syndrome, cryoglobulinaemia with Raynaud's syndrome and vasculitis, cold

agglutinin haemolytic anaemia, bleeding, or peripheral neuropathy with symmetric sensorimotor defects of the lower extremities (6).

### Heavy chain disease (HCD)

This is a family of rare lymphoma like disease that produce a monoclonal immunoglobulin devoid of light chains (6), involving the three main immunoglobulin classes:  $\alpha$ -HCD (most common),  $\gamma$ -HCD, and  $\mu$ -HCD (rarest) (11). The diagnosis of HCD depends on the detection of the structurally abnormal immunoglobulins in the patient's serum or urine.

### Leukaemias

Monoclonal proteins have been found in the serum of patients with chronic lymphocytic leukaemia (12), but identification adds little to the diagnosis or prognosis for these patients (1).

## Protein infiltrative and deposition disease

### Amyloidosis

Primary amyloidosis is one of several forms of amyloidosis, but it is the only form associated with monoclonal gammopathy (13). In this condition a monoclonal protein is produced that leads to a fibrillar protein deposition in multiple organs (6). Although a monoclonal light chain will be found in the serum or urine in nearly 90% of patients with amyloidosis, only 15% of patients will present with physical findings, therefore this presents a diagnostic challenge (14). Amyloidosis develops in approximately 10% of myeloma cases (15) and rarely in patients with WM (16). Confirmation of amyloidosis is made by examining tissue biopsies, to determine the presence of amyloid which is demonstrated by positive staining to Congo red and green birefringence under polarised light (17).

### Immunoglobulin deposition disease

This is a very rare disease, in which approximately 70% of affected patients have a monoclonal protein in their serum or urine (18). Major complications are nephrotic syndrome and renal failure due to extensive deposition of monoclonal protein in the glomeruli, but other organs affected include the heart, the liver, and the lens (6). The definitive diagnosis is made by the immunohistological analysis of tissue from the affected organ using immunoglobulin chain-specific antibodies (18).

## Miscellaneous disorders

### Monoclonal gammopathy of undetermined significance (MGUS)

Until 1984 this was known as 'benign' monoclonal gammopathy (19). A low-level monoclonal protein is present in 1% of people over the age of 50, and 3% of people over the age of 70 (20). MGUS denotes the presence of a monoclonal protein in patients without evidence of myeloma, amyloidosis, lymphoproliferative disorders, or related conditions (8).

### Transplant related monoclonal gammopathy

Patients who have had renal, liver and bone marrow transplants have a higher occurrence of monoclonal bands than the general population (8). These usually last between 2 and 6 months and are most commonly low level IgG (6).

### Transient bands

The occurrence of several small bands (and occasionally larger ones) may be of doubtful significance and can occur in acutely unwell patients. These transient bands are probably a result of restricted early antibody response (21).

### Biclonal gammopathies

Among cases of gammopathy, the occurrence of biclonal bands is approximately 1% (22). The clinical features of biclonal gammopathy are similar to monoclonal gammopathy (4,8). Triclonal gammopathy has been reported, but it is very rare (23).

## Detection of serum monoclonal proteins

### High resolution electrophoresis

The principle of electrophoresis is based on a sample being applied to a support medium, which has a negative charge in relation to the buffer it is placed in. The buffer cations flow toward the cathode creating endo-osmotic flow. The separation of the proteins is dependent upon the isoelectric point of the molecule, the concentration of the electrolytes and pH of the buffer, the temperature of the gel during electrophoresis, the characteristics of the gel, and the amount of current applied (24). Monoclonal proteins are recognised as an area of restricted migration on an electrophoretic strip or a "spike" on a densitometric tracing. Interpretation can be confusing due to the presence of bands that are not monoclonal proteins. These include increased transferrin, increased  $\beta$ -lipoprotein, fibrinogen, or CRP (1).

Electrophoresis also has a key role in offering clues towards the direction of further investigation in a number of other clinical situations. For example, an acute inflammatory response (elevation of  $\alpha_1$ ,  $\alpha_2$  and  $\beta$ -globulins), a neoplastic process (elevation of  $\alpha_2$ -globulins and a reduction in serum albumin), chronic disease which may include autoimmune or chronic infection (polyclonal elevation of  $\gamma$ -globulins), chronic liver disease ("beta-gamma" bridging due to polyclonal increase in IgA) (25).

High resolution agarose gel electrophoresis can separate up to 12 bands (26), and is the method of choice for routine serum electrophoresis (27). This technique has replaced previous less sensitive low-resolution techniques such as cellulose acetate (28), which in turn replaced much earlier filter paper methods (4). High-resolution electrophoresis of monoclonal proteins is accepted as a method that is sensitive, rapid, reliable and inexpensive, and is particularly more sensitive for the screening of small monoclonal proteins (12,29). Recent studies comparing manual and newer semiautomated techniques have shown good correlation, but there continues to be areas where difficulties may be encountered with the detection of bands by electrophoresis (30). Monoclonal IgM has been reported to be more difficult to detect due to it often having "mid-gamma" mobility that may be masked by polyclonal IgG (21), while low level IgA can also be difficult to detect due to blending with  $\beta$ -globulins (6).

Nephelometric or turbidimetric methods are routinely used for the quantification of immunoglobulins since the techniques are easily automated but the concentration can be over or under estimated. This is because the monoclonal protein may react incompletely with the anti-serum if the protein has limited or incomplete antigenic determinants or the protein may behave peculiarly compared with the calibrator (1). Because of these peculiar immunological properties routine electrophoresis combined with densitometry is the recommended method of quantitating monoclonal proteins, although the measurement of the monoclonal protein may be higher because polyclonal immunoglobulins of the same class are included (6).

### Capillary electrophoresis

This is an emerging technique with the Beckman CZE 2000 being the first commercially available system (31). Serum is injected into narrow fused-silica capillary tubes, in which the negative charge results in a strong endo-osmotic flow that pulls serum proteins toward the cathode, with gamma globulins migrating most rapidly. Peptide bonds

are measured by their absorbance of ultraviolet light (~200 nm) at the cathodal end. Capillary electrophoresis has been reported to improve the resolution of serum protein bands, whereas automation permits processing of up to 50 sera per hour (32).

The typical serum pattern in capillary electrophoresis is very similar to those seen on agarose, although the gamma zone appears to be somewhat shorter (31). Although excellent resolution has been reported, it has also been reported that large monoclonal proteins migrating in the slow gamma area have been missed. These can be detected by changing the buffer, but only one buffer is routinely used. Also some false positive results have been reported that have been presumed to be due to the presence of substances that absorb at 200 nm (26).

#### **Kappa/lambda ( $\kappa/\lambda$ ) ratios**

Kappa/lambda ratios used in conjunction with immunoglobulins have been proposed as an alternative technique for detecting monoclonal proteins, in that an altered  $\kappa/\lambda$  ratio may suggest the presence of a monoclonal protein. However, in general this technique is not recommended, particularly when low level monoclonal proteins are involved since the ratio may still be within normal limits (21). Furthermore, this approach will not recognise biconal monoclonal proteins each with differing light chains, since the ratio may remain normal (24).

## **Characterisation of monoclonal proteins**

### **Immunofixation**

Immunofixation was first described by Alfonso in 1964 (33), but it was not introduced as a routine procedure until 1976 (34). The diluted sample is separated by electrophoresis and then antisera are applied (normally anti-IgG, IgA, IgM,  $\kappa$  and  $\lambda$  to the appropriate lanes and incubated. Unprecipitated proteins are washed away with buffer and the precipitin bands are stained. If this does not adequately identify a protein, additional antisera against IgD and IgE should be used (26).

Immunofixation is more sensitive than protein electrophoresis techniques with a detection limit down to ~0.25 g/L. Immunofixation has been reported to be more sensitive for the detection of low level monoclonal proteins particularly IgM on a polyclonal background (35), that may have been missed if electrophoresis alone was used. However, the analytical sensitivity of immunofixation contributes very little to the identification of serum monoclonal proteins where tiny components are often of little clinical significance and their identification adds to an unnecessary additional workup (1). The main difficulty with immunofixation is determination of the optimal dilution at which to detect the presence of a small monoclonal protein or a massive increase in immunoglobulin (either monoclonal or polyclonal). A small monoclonal protein could be missed if too large a dilution of the patient's serum is used, while if a massive increase of immunoglobulin is present, one could have antigen excess effect where small immune complexes were washed away. The simplest way to estimate the dilution is to look at the protein electrophoresis result (26). Immunofixation is the most reliable means of characterising a biconal (or rarely, triconal) monoclonal protein, although care must be taken to exclude monomers, aggregates and polymers of a single monoclonal protein. For example, if two IgA peaks having the same class of light chain were present, the two components most likely represent monomers and polymers of a monoclonal IgA (24). When interpreting the immunofixation, if there is an area of precipitation on each lane, the possibility of the presence of a cryoglobulin must be considered and the sample should be retested following reduction with

$\beta$ -mercaptoethanol. The patient should also have serum collected and separated at 37°C to be forwarded for testing for cryoglobulins (60).

### **Immunosubtraction**

Following capillary electrophoresis, immunosubtraction can be performed to characterise the monoclonal peak. This process involves incubating the serum with Sepharose beads coupled with anti-IgG, IgA, IgM,  $\kappa$  and  $\lambda$  antisera. After incubation the supernatants are reanalysed to determine which reagent has removed the monoclonal peak (24). This method has the distinct advantage of being readily automated and is considered to be a reliable method to detect obvious monoclonal proteins (32). It has the disadvantage of being no more sensitive for small bands than capillary electrophoresis, and because the background is not subtracted out there is no improvement in contrast. Sourcing antisera has also proved to be problematic because reagents for identifying IgE and IgD monoclonal proteins are not readily available (26).

### **Nephelometry**

An alternative approach for characterisation has been proposed using  $\kappa/\lambda$  ratios in conjunction with individual immunoglobulin quantitation. But this has been demonstrated to be insensitive, particularly for low level monoclonal proteins. Also, falsely positive results were found in patients with hyper- or hypogammaglobulinaemia and therefore it is not recommended (21). Another strategy proposed to avoid the use of immunofixation has been the use of high resolution electrophoresis in conjunction with immunoglobulin quantitation to characterise the monoclonal protein. This method was demonstrated to reduce previous use of immunofixation by 88% (29). But this method has only been considered valid in the presence of an obvious monoclonal protein on the electrophoresis gel. Measurement of immunoglobulins is not an adequate screen in the absence of an abnormal serum pattern on electrophoresis. This method should only be considered as an adjunctive method to characterise monoclonal proteins and not as a screening test (26).

### **Immuno-electrophoresis**

Immuno-electrophoresis was the original technique used to characterise monoclonal proteins, but most laboratories no longer use this technique (24). It is less sensitive, slower, and often more difficult to interpret than immunofixation or immunosubtraction (26).

### **Urine analysis**

Urine electrophoresis is usually requested for two main reasons - for the assessment of renal dysfunction, and the detection and quantitation of free light chains (FLC, Bence Jones protein) (36).

In glomerular disease larger proteins ( $M_r >66000$ ) pass more freely than usual through the leaky glomerular basement membrane. The excreted protein is mostly albumin without small globulins which are reabsorbed by the tubules, but the larger proteins are seen when the threshold for tubular reabsorption is exceeded. While tubular proteinuria results in the urine containing elevated levels of small proteins ( $M_r <66000$ ) including polyclonal light chains and  $\beta_2$ microglobulin. A mixed pattern is seen with combined glomerular and tubular damage (38,39).

Dr Henry Bence Jones and others first described FLC in 1845 (37). Bence Jones proteinuria is an "overflow proteinuria" associated with immunoproliferative disorders and is characterised by the spillover of excess FLCs into the urine (38). The presence of FLC does not necessarily imply the presence of renal damage. FLC are usually  $M_r$  22000 monomers or  $M_r$  44000 dimers and as a result easily pass



through the glomerular basement membrane (1). One of the major causes of renal insufficiency in myeloma patients is "myeloma kidney" which is characterised by the presence of large, waxy, laminated casts in the distal tubules and collecting ducts. These casts consist of precipitated monoclonal light chains that cause the renal tubules to dilate and the entire nephron to become distorted and non-functional (40). Although a 24 hour urine collection allows for better longterm monitoring of FLC excretion, a random urine sample is most commonly used for the detection and characterisation (37).

### Detection of free light chains

Several methods of detection of FLCs have been described but to date no single method is considered sufficient on its own. Historically, Dr Bence Jones' thermal denaturation test was used, but this method has been demonstrated to produce false-positive and false-negative results (4). Dipsticks are used in many laboratories as a convenient screen for protein but these are based on tetrabromophenol which FLC do not react with, so therefore should not be used (36).

### Urine electrophoresis

FLC can be detected by electrophoresis on agarose gel followed by staining with a protein specific stain such as Amido Black, Coomassie Brilliant Blue, or Ponceau S (32,38). These methods are reported to be capable of detecting FLC at 10 mg/L (37). Ultra sensitive methods with a detection limit of 2-10 mg/L, such as colloidal gold and colloidal silver staining, are available. While these methods have no concentration step, the techniques are more demanding and the electrophoresis patterns are not similar to those of traditional methods, so may cause difficulties in interpretation (37,38,41). Although urine electrophoresis is a useful screening technique, it fails to detect low levels of FLCs particularly in the presence of a masking glomerular type proteinuria (38).

Most methods require a prior concentration step, but there is no standardisation of this and it is time consuming and expensive. Urines are concentrated 100- to 150- fold of the total protein value (1), but this approach can cause problems since there is not necessarily any correlation between total protein and the urine FLC level (34). Pre-concentration is associated with a loss of low  $M_r$  proteins which can lead to a 30% overestimation of the albumin fraction (36) and may cause some FLCs to polymerise and aggregate (38). Some semi-automated systems, such as the Sebia Hydrasys, employ methods which use unconcentrated urine for screening of FLC which avoids the problems associated with the pre-concentration step. The sensitivity is increased in this system by using high-resolution gels and acid violet as the stain.

### Sodium dodecyl sulphate - agarose gel electrophoresis (SDS-AGE)

SDS-AGE separates proteins based on molecular weight (32). It has the distinct advantage of differentiating proteinuria of glomerular, tubular, or mixed origin (36). SDS-AGE has the advantages of giving a better indication of the site of protein loss, is considered more sensitive for detecting FLC, and dispenses with the concentration step (32). While a disadvantage is that SDS-AGE does not definitively distinguish between monoclonal and polyclonal light chains because they have the same molecular weight (32). This is particularly significant in patients with myeloma who may have tubular damage and excrete a complex mixture of monoclonal and polyclonal light chains (36). SDS-AGE is generally considered to be more technically demanding but this has been reduced somewhat by the availability of commercial kits (38). A SDS-AGE method has been described which contains the reducing agent  $\beta$ -mercaptoethanol, which exploits protein denaturation that can

minimise or eliminate the tendency of FLC to polymerise and form protein complexes that can result in multiple anomalous bands, also referred to as the "ladder pattern". Under the reducing conditions of  $\beta$ -mercaptoethanol, FLCs appear as a single dense band at Mr 22000 and in most cases are not confused with polyclonal light chains, and in some cases may be more sensitive than immunofixation (36).

### Capillary electrophoresis

Capillary electrophoresis has been widely reported for the use of serum and it is potentially suitable for use in urine, but awaits validation (37). It still requires perfection for analysis of urine (1) because even though some good correlations have been obtained between capillary electrophoresis and routine urine electrophoresis for the determination of albumin and FLC, these require significant pretreatment (38).

### Nephelometry

Nephelometry has been proposed because in one study a significant correlation was found between FLC detected by nephelometry and SDS-AGE (36). In the past there have been difficulties with specificity and measurement errors due to polymerisation, and monoclonal antibodies have been difficult to develop (42). Nephelometry is not recommended for initial screening and quantitation due to over or under estimation (36), but FLC immunoassays are considered to be most useful in detecting and monitoring patients with Bence Jones nonsecretory myeloma (43). Recently latex based nephelometric and turbidimetric methods, such as the Freelite assay performed on the Beckman IMMAGE, have become available for the measurement of serum total FLC and FLC ratios. This method is reported to be more accurate since there is no effect from renal tubular metabolism and have lower limits of sensitivity than traditional methods. It has the advantage of being automated so is therefore quicker to perform (59).

### Characterisation of free light chains

Urine immunofixation is the most commonly used technique for the characterisation of monoclonal bands in urine as the test is 10-fold more sensitive than electrophoresis. The principle of this technique remains the same as for serum immunofixation (37), and can achieve sensitivity to 50 mg/L (34).

There are variations on how urine immunofixation is routinely performed. The urine can be immunofixed in a similar manner as a serum sample, using monospecific antisera for IgG, IgA, IgM,  $\kappa$  and  $\lambda$ , where the FLC can be recognised as a band of migration reacting only with the light chain antisera with no corresponding heavy chain (34). However, since immunofixation uses antisera containing free and bound light chains, intact monoclonal immunoglobulins comigrate with FLC and therefore can not be distinguished, and it may also encounter difficulties in detecting FLC in the presence of high levels of polyclonal light chains (38). Another approach is to perform routine electrophoresis on the urine sample in conjunction with immunofixation for only  $\kappa$  and  $\lambda$  light chains, if this is positive then the sample can be retested by immunofixation to exclude intact immunoglobulins (32).

The "ladder" pattern caused by polymerisation of polyclonal light chains, due to the pre-concentration of the urine, continues to be a problem confusing the immunofixation pattern.

## 6. Screening and Diagnosis

Serum electrophoresis is recommended when myeloma, WM, amyloidosis or a related condition is suspected. In addition

electrophoresis has been recommended in any patient with unexplained weakness or fatigue, anaemia, elevation of ESR, unexplained back pain, osteoporosis, osteolytic lesions or fractures, hypercalcaemia, Bence Jones proteinuria, renal insufficiency, recurrent infections, unexplained sensorimotor peripheral neuropathy, carpal tunnel syndrome, refractory congestive heart failure or cardiomyopathy, nephrotic syndrome or renal insufficiency, orthostatic hypotension, malabsorption, weightloss, light-headedness or syncope, change in voice, increased bruising, bleeding, or steatorrhea (24). By following these recommendations, electrophoresis would become essentially a screening test and laboratories may have difficulties coping with performing large numbers of this test. In practise, diagnosis of plasma cell disorders and related conditions is dependent upon a number of tests and therefore a multidisciplinary approach is required. These include biopsies, clinical assessment, radiology, and a laboratory wide assessment including blood counts, calcium and renal function tests, urinalysis, and immunoglobulin measurement (24).

The detection of a previously undetected monoclonal protein can cause somewhat of a management challenge for a GP, particularly if the band is relatively small and the GP has been provided with an identification of the peak by immunofixation. In routine practice the majority of bands are small and unless the patient has particularly suspicious clinical features the patient could continue to be managed by the GP. A reasonable recommendation the laboratory could make to the GP is to perform a repeat serum EPP in several months (for example 3-12 months), since a number of these may be of "transient nature". At the time of initial detection it could be argued that immunofixation need not be performed on this subset of monoclonal bands, because characterisation of the band at the time of detection offers no diagnostic value and may on cause the GP uncertainty in management of the patient. But conversely, immunofixation is recommended when electrophoresis is negative when there is adequate suspicion of a malignant monoclonal gammopathy (27), because immunofixation is more sensitive than electrophoresis for the detection of monoclonal proteins. For instance, to diagnose amyloidosis, electrophoresis and immunofixation are the best noninvasive tests, since nearly 90% of patients with primary amyloidosis have a monoclonal light chain present in the serum or urine. Furthermore, immunofixation should be performed on both serum and urine because the monoclonal protein is absent from the serum in one third of patients and the peak is often overlooked in electrophoresis (14), while heavy proteinuria frequently seen in amyloidosis can obscure the presence of FLC (13).

This highlights the need for communication between the clinician and the laboratory. The laboratory must be aware of patients with whom the clinician has a high suspicion of a malignant monoclonal gammopathy who may require further laboratory investigation following a normal electrophoresis scan. Effective communication between the laboratory and clinician can allow the optimal use of laboratory while investigating monoclonal gammopathies in a manner that is medically sound (44).

## 7. Managing Patients with Monoclonal Gammopathy

The process of diagnosing myeloma is changing over time. Whereas in the 1960s 68% of patients presented with bone pain, this had dropped to 37% in the 1980s (5). Presumably due to the more frequent requesting of electrophoresis and the development of more sensitive electrophoresis assays, so using bone pain as a diagnostic indicator can no longer be relied on to the same extent as previously.

While it has been suggested that the most reliable means of distinguishing a benign from a malignant course is the serial measurement of the monoclonal protein (19), in reality this does little to provide an initial diagnosis and to alleviate the immediate concerns of both the clinician and the patient. The size of a monoclonal protein may make a clinician more suspicious of a malignant process particularly if the peak is relatively large. What is generally more important is the presence of peak in conjunction with clinical findings and the patients symptoms. While some specific criteria for diagnosis have been published (1,4,6), there is some disagreement and uncertainty when trying to make a definite diagnosis of a particular monoclonal gammopathy.

It is important to accurately diagnose myeloma, be able to differentiate this from smouldering myeloma, WM and MGUS. It should also be kept in mind that the initial workup is dependent upon the hypothetical relative risk. Therefore patients who are in the low-risk category for transformation should avoid invasive and expensive examinations, such as bone marrow aspirations and biopsy, skeletal survey, or MRI scans (45). Patients without myeloma or WM may be safely observed off chemotherapy since there is no advantage in early treatment and unnecessary treatment can cause increased morbidity or mortality (46).

The point at which MGUS evolves into WM remains ill-defined and is a distinction made clinically. WM has three important distinguishing features: pulmonary involvement, peripheral neuropathy, and hyperviscosity syndrome (2). The light chain of the monoclonal IgM is  $\kappa$  in 75% of patients and the level of monoclonal protein is usually greater than 30 g/L. FLCs are present in the urine in 40% of cases but usually measures less than 1 g/day (16). At present there are no uniform criteria for the diagnosis of WM, although the following criteria have been proposed: 1) presence of monoclonal IgM of at least 10 g/L; 2) bone marrow B-lymphoid cell infiltration greater than 30% of total nucleated marrow cells; 3) need for therapy due to signs or symptoms of the disease within 6 months of diagnosis (16).

Urine FLCs are reported to be present in 60% or more of all myeloma cases (47), and renal impairment is observed in about 50% of myeloma patients due to light chain excretion during the course of their disease and is associated with poor prognosis. Since light chain excretion has been described as an essential factor contributing to renal failure (36). Methods for the measurement of FLCs have been reported to detect as low as 1 mg/L. While this is impressive at an academic level, this in fact reduces the specificity of FLCs as a marker for malignancy since it has been reported that levels of <60 mg/L can be observed in benign processes (37). For the purpose of writing this paper, seven clinical haematologists were contacted regarding their use of FLC. In general terms they were asked how the presence of FLC alters management decisions in the diagnosis of patients with monoclonal gammopathy. All the haematologists responded that they would have increased level of suspicion of myeloma, when a patient has FLC in conjunction with a monoclonal protein. The presence of FLC would generally lower their threshold for further testing as they would be more suspicious of myeloma. It is interesting to note that only three of the haematologists provided an absolute value that would alter management of their patients. Two of the haematologists place more value on the trend shown by the FLC quantitation, while the remaining two haematologists placed more diagnostic value on other clinical findings. The presence of FLC would make all the haematologists more fastidious in monitoring the patient's renal function.

It is important to continue to monitor patients with MGUS for the rest of their life. Although this was initially considered to be a benign disorder (48), it is now known a substantial proportion will develop a malignant monoclonal gammopathy (49). At the Mayo Clinic, MGUS patients have been followed for 24 to 38 years. After 25 years, 40% had developed myeloma, lymphoproliferative disorders, amyloidosis or a related disease (20). In a small New Zealand study that followed eleven MGUS patients for 31 years, 7 (64%) progressed to a malignant monoclonal gammopathy (50). It is interesting to note that the risk of malignant transformation was not dependent of the type of monoclonal protein (IgG, IgA or IgM), the level of the monoclonal protein, the presence of FLC or the percentage of BMPC (49,8). MGUS patients that do not progress to a malignant monoclonal gammopathy have increased mortality above that of the general population with a standardised mortality rate of 1.7. This may be explained by the concept of "confounding by indication", which implies that electrophoresis is performed on a selected group of patients and that the symptoms that led to that request must be ascribed to a unrelated coexisting disease (51). It is important this concept is considered by the clinician.

## 8. Other Laboratory Methods

In addition to serum and urine proteins determinations for managing monoclonal gammopathies, several other methods can be used. Generally, these are used to differentiate monoclonal gammopathies, and to detect or predict progression.

### • Interleukins

Interleukin-6 (IL-6) is a potent plasma cell growth factor (52). Myeloma cells have been shown to proliferate in response to IL-6 while normal cells do not have the same response (46). Interleukin-1 $\beta$  (IL-1 $\beta$ ) is produced by the plasma cells of virtually all myeloma patients but is not detectable in the plasma cells of most MGUS patients. Since IL-1 $\beta$  has potent osteoclast-activating factor (OAF) activity, it may be responsible for the presence of bone lesions (46).

### • Lymphocyte markers

Two clearly defined and distinct plasma cell populations have been identified in MGUS and myeloma. One population was polyclonal while the other plasma cell subpopulation was clonal with each demonstrating different lymphocyte marker expression. Only 1.5% of patients with MM had more than 3% of normal polyclonal plasma cells, but 98% of patients with MGUS had more than 3%. This demonstrated significant differentiation between MGUS and myeloma (8).

### • Plasma Cell Labelling Index

The plasma cell labelling index represents the percentage of plasma cells in the S (proliferative) phase of the cell cycle and requires the incubation of a bone marrow specimen with radioisotope. Studies have shown that a labelling index between 1% and 3% is typically present in overt myeloma, while values consistently below 1% are found in MGUS. A high labelling index appears to be a reliable diagnostic indicator of myeloma and also correlates with progressive disease. However, a normal labelling index does not rule out myeloma. Unfortunately, plasma cell labelling index determinations are not widely available outside of large research-orientated institutions (45).

### • $\beta_2$ -microglobulin and CRP

$\beta_2$ -microglobulin is often used to help manage myeloma, since it reflects overall plasma cell tumour burden and correlates well with

prognosis. But unless the  $\beta_2$ -microglobulin level is extremely high, it is not helpful in distinguishing myeloma from MGUS, since there is considerable overlap (45). It has been suggested that  $\beta_2$ -microglobulin and the plasma cell labelling index which are independent variables can be used together to identify mean survival times in patients with myeloma. As plasma cell labelling is not readily available, CRP has been recommended as a simple and valid alternative (5).

### • Cytogenetics

Cytogenetic studies have been performed on BMPC of MGUS patients that have demonstrated several abnormalities. This has been hypothesised to be due to the gradual acquisition of cytogenetic changes that may develop into a malignant monoclonal gammopathy (53).

## 9. Use of Protein Studies for Monitoring Treatment

At present, cure is a realistic goal for only a small minority of patients with myeloma. The current treatment options include interferon (54), standard chemotherapy, or high-dose chemotherapy followed by autologous transplantation regimens with bone marrow or peripheral blood stem cells (55). In spite of these treatments only approximately 5% of patients will be alive 10 years after diagnosis (3), and the mean survival is three years (56). For the vast majority of myeloma patients, the rational goals of treatment are meaningful prolongation of life with durable relief of pain and other disease symptoms, and protection of a normal quality of life for as long as possible. These are usually achieved through reduction of the myeloma tumour burden by establishing a plateau phase and delaying disease progression. Treatment beyond the plateau has no reported benefits, but prolonged therapy carries a substantial risk of leukaemia and myelodysplastic syndrome (55). Complete response is currently defined as a serum or urine monoclonal protein that is undetectable by sensitive assays, usually immunofixation. In addition, the percentage of BMPC must be reduced to normal levels of less than 5%. Partial response is >90% reduction in monoclonal protein level (56). In reality, disappearance of the band occurs in only 3% of patients but a 50% reduction is considered a good clinical response. In patients who do response to chemotherapy, FLC excretion is reduced more rapidly than the serum monoclonal protein, with a 50% reduction occurring in less than 2 months as compared with about 3 months for the serum monoclonal protein. But the reduction of the monoclonal protein may be misleading, because several biologic factors influence the correlation of the monoclonal protein and tumour burden. For example the half-life of the IgG<sub>3</sub> subclass is shorter than that of other IgG subclasses and may affect monoclonal protein reduction after chemotherapy. Furthermore a relapse phase is frequently revealed by increased numbers and/or size of bone lesions, with out a corresponding monoclonal protein increase (5).

Some patients with WM have shown good response to plasmapheresis (since 80% of the IgM is intravascular), high-dose intravenous immunoglobulin, chemotherapy and interferon. In these cases partial response is defined as at least a 50% decrease in the level of monoclonal IgM with more than a 50% reduction of bone marrow lymphocytosis and organomegaly (57).

Amyloidosis has been a difficult disease to treat, although recent developments in the use of melphalan and autologous stem cell transplantation has been promising. However the mortality rate using this approach averages 27% which is still unacceptable. In patients treated successfully, complete response is determined by the absence



of previously detected monoclonal plasma cells, and absence of a monoclonal protein in the serum or urine. Patients who have  $\kappa$  clones are reported to be more likely to respond than those who have  $\lambda$  clones (58).

## 10. Conclusion

Monoclonal gammopathies can be difficult to make a definite diagnosis, since there is often considerable overlap between disease entities. While overt myeloma is probably most clearly defined, other conditions are much less so. It is important the personnel performing the laboratory tests have an understanding of the conditions they may be detecting, while also understanding how different approaches to testing methods can make the results more accurate.

For the medical laboratory to provide information that will most help the clinician when investigating monoclonal gammopathies, clear and open communication is required. It is important in the screening and diagnostic stage, in that the clinician must communicate their degree of suspicion of a monoclonal gammopathy. Doing so allows the appropriate degree of testing to be undertaken and more intensive investigations performed when justified by the clinical details. Furthermore, the clinician and the laboratory can continue that partnership when managing a patient with a monoclonal gammopathy. For example, the laboratory can help determine if an indolent condition (such as MGUS or smoldering myeloma) has progressed to an overt malignant process, which may be the indication for the clinician to begin treatment. The clinician can use laboratory information to determine the prognosis and appropriate treatment regime, and the patient's response can be monitored.

For the medical laboratory to operate in this partnership effectively, it is essential the laboratory has a sound knowledge of the conditions associated with monoclonal gammopathies, appropriate testing regimes, and is able to assist in the patient's management. Only laboratory staff with sound knowledge, working in conjunction with the clinician, can provide the best available outcome for the patient.

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# The implications of methicillin resistant *Staphylococcus aureus* and why we should keep it out of hospitals

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## Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) are all resistant to the  $\beta$ -lactam group of antibiotics. Horizontal transfer of the *mecA* gene from MRSA to methicillin sensitive *Staphylococcus aureus* (MSSA) means that more *Staphylococcus aureus* (*Staph aureus*) will become resistant to these antibiotics. If the strain is virulent and perhaps resistant to conventional antibiotics, treatment of an infection caused by these strains is difficult. This means that more complex antibiotics (which may promote further resistance developing) will need to be used, and more time in hospital is needed, resulting in more money being spent. Epidemiological studies have shown that MRSA has spread all over the world, and New Zealand has been able to monitor both the community strains (CMRSA) and the multi-resistant strains present in the population. A good understanding of the role of MRSA in hospitals is needed.

A discussion on what may influence the outcome of *Staph aureus* infections is presented. Factors such as virulence, antibiotic use and transmissibility of strains of MRSA and MSSA are discussed. It appears that although all can contribute, none are alone in promoting MRSA or MSSA infections. The one factor that seems to predispose MRSA morbidity is the state of the host. Comparisons of MRSA and MSSA show that there is no difference between these two general strains that indicate that one is more virulent or contagious than the other.

The fact that MRSA can outlast MSSA in the environment indicates that hospital cleanliness is required to prevent the spread of MRSA. Good screening methods for MRSA carriage in in-coming patients and new staff members can significantly reduce the numbers of MRSA infections in hospitals, and good hand-washing techniques and isolation of infected patients can keep transmission levels down.

## Key Words

MRSA, community acquired MRSA, virulence, hospital acquired infection, epidemiology, antibiotic resistance, *mecA*.

## Introduction

*Staphylococcus* is a genus of about 32 species and 15 subspecies, some commensal, some pathogenic. It is a gram positive, catalase positive, non-motile, non-spore forming, generally facultative anaerobe, first named in 1883 by Sir Alex Ogston (1). He attributed this organism as the cause of inflammation and suppuration. The most clinically significant species is *Staphylococcus aureus* (*Staph aureus*), whose main habitat is the nasal membrane and skin of warm-blooded animals. Infections may be mild, (e.g. skin infections, food poisoning) or severe, causing pneumonia, sepsis, osteomyelitis, infectious endocarditis. Before the advent of antibiotics, common boils and abscesses often went on to cause septicaemia, with 90% proving fatal (2). During that time, the streptococcal genus was the prime cause of hospital-acquired infections (HAI). When penicillin and other crude antibiotics were developed in the 1940s, the sensitive streptococci became less of a problem, and *Staph aureus* soon took its place as the major cause of HAI (3). It was initially sensitive to penicillin and easy to treat, but by 1944-45 resistance to penicillin was up to 14% (2).

During the 1950s a pandemic was caused by a penicillin resistant

strain of *Staph aureus* (named the H-Bug) that caused hospital wards worldwide to close to new admissions (4). It was this pandemic that prompted both the formation of infection control teams in hospitals and the development in the 1960s of new types of  $\beta$ -lactamase stable penicillins, the  $\beta$ -lactams, of which methicillin is one. With this development came the almost simultaneous evolution of a new strain of *Staph aureus*, one that was resistant to methicillin, and consequently labelled MRSA (methicillin resistant *Staph aureus*) (2,5). It was gradually found in hospitals all over the world.

MRSA is only a problem for people who are already sick or in some way immunocompromised. These people have lowered resistance to bacteria and they can become very ill with bacteria that would otherwise not cause any illness. The major concern with MRSA is that it is resistant to many common antibiotics, with some being resistant to most antibiotics that are available. This means that if serious illness is caused by MRSA, then stringent nursing methods and complex antibiotics have to be used. These same antibiotics can encourage the same organism to become resistant to them, especially if not treated completely. Herein lies the problem - if the bacteria become resistant to the antibiotics that are used to treat it, what is left to treat it with? Is a monster being created? Should hospitals and secondary health care facilities be kept free from MRSA in an effort to minimise the risk of more resistant strains of MRSA emerging? Are there differences in the various types of MRSA around?

The epidemiology of this organism and all its strains points to the ease with which it has spread throughout the world and may show that it is indeed worth the effort to keep resistance and emergence of new strains to a minimum.

## Epidemiology

In 1967-1968, 1.4% of patients in Boston City Hospital were infected with MRSA, and by 1991, 29% of hospital acquired infections (HAI) in the United States were caused by MRSA, which were then disseminated into the community (2). In Nigeria, between December 1994 and April 1995, out of 100 *Staph aureus* isolates, 27% were methicillin resistant, 27% showed borderline resistance and 46% were sensitive to methicillin. Of those showing resistance, 41% were from inpatients while 59% came from outpatients, indicating just how successful it was at surviving out in the community (6). In Japan, 60-90% of *Staph aureus* infections are caused by MRSA, and in fact it is seen as being as familiar to the public as AIDS is (7). In a month-long survey of 830 French hospitals during 1996, it was found that *Staph aureus* accounted for 16% of all HAI and that 57% of these were MRSA (8).

Different strains now predominate in various countries and these seem more able to cause infection - EMRSA-15 (Epidemic strain of MRSA) in the United Kingdom (UK), the Iberian strain in Europe and the Brazilian epidemic clone (BEC), which may soon be the most predominant one in Europe as well as in South America (9,10). At the other end of the scale are countries like the Netherlands, where MRSA prevalence is low ( $\leq 1.5\%$ ) (11). In the Pacific region, which is more geographically isolated, the pattern is the same, with Australia isolating its first MRSA in 1965 (12), and New Zealand in 1975 (13).

From this information it can be seen that by the late 20th Century,



MRSA was causing significant hospital-acquired infections. These are infections that patients get within the hospital setting and there are set criteria governing the labelling of this type of infection (14).

EMRSA-15, one of the Epidemic MRSA strains currently predominant in the UK, was introduced into New Zealand hospitals in 1995 by staff originating from British hospitals (15). This strain is characterised by showing resistance to ciprofloxacin and erythromycin, as well as the B-lactam group of antibiotics. Various outbreaks of this strain have occurred and the rate of isolation is rising steadily, with 39.5% of all MRSA strains being EMRSA-15 in 2002 (16).

Another multi-resistant strain is making its mark in New Zealand hospitals, and that is an Australian import designated AKh4. It is typically resistant to ciprofloxacin, clindamycin, cotrimoxazole, erythromycin, gentamicin and tetracycline (17). It has become clear that these, and other new, multi-resistant strains will become more prevalent if strict infection control procedures are not taken. One of these new strains, in particular, was sensitive to vancomycin and fusidic acid only, leaving no room for compromise (personal communication).

New Zealand, as a nation, is made up of indigenous people (the Maori) and immigrants. Initially, these immigrants were of Anglo-Saxon origin (i.e. from the UK) and this group were followed by people from Europe, the Pacific nations and Asia. This, in a way, is reflected in the predominant strains of MRSA found in the region. MRSA first came to New Zealand from the UK and the strains found here now mimic those found in the UK, albeit with a time delay. We also harbour the dominant patterns found in Western Samoa (WSPP1 and WSPP2) (18). This may all indicate that the genetic make-up of a population predisposes what types of MRSA could become endemic. If New Zealand had a high level of Brazilian immigrants, perhaps the predominant strain would be their BEC strain? At the moment, the national surveillance centre for New Zealand, the Institute of Environmental Science and Research Limited (ESR) does not specifically look for these strains, so no conclusion can be reached on this.

In addition to the strains that have recently been imported from abroad, New Zealand, like the rest of the world, has its own 'Community Strains' (eg WSPP1 and WSPP2) as well as 'Epidemic Strains' (eg EMRSA-15). A 'Community Strain' (CMRSA) is the term used to define an MRSA that may be found on people with no history of hospitalisation, or those that are isolated from people within 72 hours of hospitalisation. Epidemic strains are those that spread readily in and between hospitals. Both of these groups consist of *Staph aureus* that are resistant to methicillin and other B-lactam antibiotics. On the whole, however, the community strains appear to be less resistant to antibiotics other than the B-lactams and are consequently easier to treat if deemed necessary (14).

In New Zealand, the WSPP1 and WSPP2 strains appear to be prevalent amongst, and almost particular to, the Pacific Island peoples, so much so that outbreaks within hospitals can occur if a Pacific Islander, colonised with these particular strains, shares a cubicle with other Pacific Islanders. The strain is not so easily transferred to Europeans, and may have something to do with the types of receptors present on the skin of differing races of people (personal communication). The WSPP1 and WSPP2 strains tend to produce skin and soft tissue infections, especially amongst the younger age group (18). These strains came to New Zealand from Western Samoa initially, but are now considered to be part of New Zealand's own environment. This is, in part, due to the large numbers of Pacific Islanders living permanently in New Zealand.

Up until quite recently, people colonised with Community MRSA (CMRSA) were not cleared of the organism (treated) unless the strain was causing infection, and this was thought to not happen very often. In other words, it was thought of as a commensal organism. However,

there is some evidence showing that some of these 'community/commensal' strains can cause life-threatening infections (19). In this case, treatment is necessary, but does run the risk of promoting further resistance of the organism. Whether non-life-threatening infections need treating or not, remains undecided. The prevalence and morbidity of CMRSA should be monitored so that a better understanding of the place of these organisms in society is achieved.

### Laboratory methods

There are many excellent papers reviewing methods available for isolation, and epidemiological typing, of MRSA and MSSA, and so this will not be discussed here (5-7,12-14,18-34). Research laboratories throughout the world are working to find reproducible ways of comparing different strains of *Staph aureus* and new ideas are being tested and reported on in the literature constantly, necessitating vigilant reading.

What is needed, however, is a rapid way of showing that a person does not have MRSA. At the moment, there is a delay of about four days. In busy hospitals where bed space is critical, this is far too long. The need for this research was apparent in a paper written 12 years ago, yet this delay still exists (35).

New Zealand is relatively lucky as the majority of its MRSA strains are able to be phage typed; other countries are not so lucky. Antibigram patterns are able to give a loose indication of the strain of MRSA isolated, and this is used in most diagnostic laboratories routinely.

### MecA

*Staph aureus* produces four major penicillin-binding proteins, PBP 1-4. These are membrane bound DD-peptidases that catalyse the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell wall. The  $\beta$ -lactam stable antibiotics (eg methicillin) are substrate analogues that bind to the PBP-active site, inactivating the eNew Zealandyme proteases. PBPs 1,2 and 3 are essential for cell growth, but as they have a high affinity for most  $\beta$ -lactam antibiotics, they are rendered inactive by these antibiotics, causing lysis and cell death (36).

Resistance to any antibiotic can be induced by the mere presence of the antibiotic. This is because the one resistant surviving bacterium can readily transmit its specific resistance property to other organisms, forming resistant populations. *Staphylococci* are well known for their ability to become resistant to some antibiotics, as are other bacteria, notably the gram-negative organisms. In general, in countries where antibiotic use is high within the general population, high levels of MRSA and other resistant organisms also occur (2).

When resistance of *Staph aureus* to the  $\beta$ -lactam antibiotics was found to be stable and becoming more common, the genomes of both MSSA and MRSA were examined to see if any stable differences between the two could be discovered; if this were the case the possibility existed that laboratory tests to differentiate the two would be developed. One such element was discovered, the gene *mecA* (36).

It is known that most of the antibiotic resistance genes for all organisms are carried by either plasmids or by mobile genetic elements. In MRSA cells, a resistance island, named *mec*, was discovered that encodes resistance to a large number of agents. The *mec* DNA contains *mecA*, (the structural gene for PBP 2a), *mecI* and *mecR1* (the regulatory elements controlling *mecA* transcription) and some *mec* - associated DNA.

In MRSA cells, PBP 2a can substitute for PBP 1,2 and 3. As it has a low affinity for binding with  $\beta$ -lactam antibiotics, PBP 2a can perform the essential functions of high affinity PBPs at concentrations of antibiotic that would otherwise be lethal. (36). This means that *mecA*

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confers upon *Staph aureus* a cell wall that is insensitive to the action of  $\beta$ -lactam antibiotics.

There are now simple ways of detecting *mecA* and it is most important that these tests are included in the laboratory identification of all suspected MRSA isolates, as some non-MRSA strains may show some resistance to methicillin (7,11,12,20-28). Strains with borderline resistance do not contain the *mecA* gene and resistance is not based on the production of PBP 2a. This knowledge helps when choosing the appropriate antibiotic for treatment (11).

The genetic difference between MRSA and MSSA is the presence of the *mecA* gene, which is present only in MRSA cells. This confers upon this strain the ability to resist all  $\beta$ -lactam antibiotics, and often many others as well. In some strains of *Staph aureus*, this is the only difference between the MSSA and the MRSA strains (30). It appears from recent work that the genetic segment that contains the *mecA* element found in CMRSA can be readily mobilized and is capable of horizontal transfer, inferring that any MSSA strain may easily become resistant to methicillin and other  $\beta$ -lactams. If the strain of MSSA is a virulent strain, treatment problems will arise when it acquires the *mecA* gene (30).

### **Possible factors that could contribute to MRSA morbidity**

Is there any one factor that can totally account for the why MRSA causes problems within the hospital setting? The following section will examine a range of factors that may be responsible.

### **Virulence factors**

When looking for differences between MRSA and MSSA, the question of whether one produces significantly more severe illnesses than the other, and whether the mortality rate is higher, arises. Looking inside the organism again, may hold the clue.

As most staphylococci are easy to cultivate in the laboratory, whole genome sequencing has been done on some representative strains of both MRSA and MSSA organisms. Using this method, over 70 virulence factors have been identified, and differentiated into four large groups: adhesions, exoenzymes, exotoxins and 'others' (37).

**Adhesions:** *Staph aureus* produces surface proteins that act as specific receptors for extracellular matrix proteins of host tissue. Collagen-binding proteins are strongly associated with osteomyelitis and septic arthritis, while the sialoprotein-binding protein is associated with initiation of bone infections. Some can initiate subacute bacterial endocarditis by mediating bacterial attachment to the extracellular matrix of the damaged heart valve, perhaps explaining why *Staph aureus* is one of the main causes of fatal bacterial endocarditis (37). The ability to adhere to and invade epithelial respiratory cells is thought to have led to the successful spread of the Brazilian epidemic clone of MRSA, found in at least five cities in Brazil, in Argentina, Portugal, and parts of Europe (9).

**Exoenzymes:** *Staph aureus* secretes many exoenzymes that degrade and digest organic compounds and macromolecules of human tissue, a process that facilitates its invasiveness.

**Exotoxins:** These include the toxic shock syndrome toxin (TSST) that causes severe menstrual and non-menstrual toxic shock syndrome, and the staph exotoxin-like protein (SET-SE), which is capable of inducing pro-inflammatory cytokine production by human peripheral blood mononuclear cells.

'Other' pathogenic factors include a diarrhoeal toxin demonstrated in Japanese MRSA strains. It causes watery diarrhoea (37).

It has been found that both types of MSSA and MRSA can produce all or some of these factors, showing that there is no difference when it comes to genetic virulence markers. These include their adherence

capacity, intraleukocytic survival, production of haemolysins and toxins, and also the outcome of experimentally induced infections (38). Hence some strains may be able to invade the skin, producing a boil, which may then go on to produce osteomyelitis, while others may not possess the appropriate factors to enable it to get past host defences in order for it to produce a fulminating infection. One study showed that MSSA was more toxic to mice than MRSA, that female mice were more susceptible to infection than males, and that castrated mice were infected at a rate in-between males and females, indicating that hormones could play a role in immunity (39). Actual practice seems to dispute this last finding (47,39).

In a study to see if epidemic strains of MRSA (EMRSA) were more virulent than non-epidemic MRSA strains, rats were injected with the predominant local EMRSA strain, inducing endocarditis. It was found that the amount needed to produce the infection was similar to the amount needed by the non-epidemic strain, suggesting that they both had the same ability to cause infections (26).

To see if there was any difference between CMRSA and hospital acquired MRSA, gene sequencing was performed on isolates from Europe, USA and the Pacific region. Two gene loci were common to all CMRSA strains from all regions: the methicillin resistance gene was found to be the shortened SCCmec type 1V element, and all contained the Panton-Valentine leukocidin (PVL) gene. This PVL gene is associated with skin infections, and occasionally, severe necrotizing pneumonia. None of the hospital acquired MRSA strains contained either the shortened SCCmec type 1V element or the PVL gene. How useful this finding is remains to be seen (19).

It is very difficult to attribute a particular virulence to MRSA, defined by morbidity or mortality, as pre-existing and underlying diseases, source of infection, shock and inappropriateness of antibiotic therapy all have an important impact on survival (38). Many studies have been done to find out if these infections are more serious than those caused by MSSA. However, there appear to be methodological shortcomings of published investigations (38,40,41).

At the Ninth International Symposium of Staphylococci and Staphylococcal Infection, held in June 2000, it was noted that the search for the "ultimate virulence-regulation gene continues" as well as the struggle to provide a vaccine (42). Until more research is published concerning any virulence differences there may be between MRSA and MSSA, no conclusions can be made as to the greater or lesser ability of different strains to produce consistently more severe infections. However, could some strains be more contagious than others?

### **Transmissibility**

The ability that all strains of *Staph aureus* (and other bacteria) have to spread easily from one person to another is known as cross-infection. The H-bug experience in the 1950s demonstrated that MSSA can be extremely contagious; possibly more than any MRSA strain so far (4).

The CMRSA strains are generally thought of as less virulent, but more contagious, than the other MRSA strains. This is particularly so with elderly people, perhaps reflecting the exposure to antibiotics and hospital life that age and frailty bring (43). It may also be because the natural barriers of the skin break down as age advances, allowing more skin colonisation. This lack of virulence could also be because these strains do not elicit a strong immunological response from the host, and, because there is little reaction to it, it may be easily passed from one person to another (personal opinion).

As noted before, different strains predominate in different countries. The BEC strain's ease of transmission may be due to its ability to adhere to the epithelial respiratory cells and so is easily passed on to



other patients; it may also be because it is a very adaptable strain of MRSA, being isolated from bronchial aspirates, surgical wounds, osteomyelitis, urinary tract infections and skin infections. It is also multi-resistant, making treatment very difficult (9,10).

One of the factors that may contribute to the ease of transmission of MRSA and MSSA is survival in the environment. The ability of *Staph aureus* to last for long periods outside the host has been known for many years. It is extremely tolerant of harsh environmental conditions and can last longer than 6-12 months (44). Studies have shown that EMRSA lasts significantly longer than sporadic or community strains of MRSA - for one to three months longer, in higher concentrations, and even in dusty environments - and that most MRSA also outlive MSSA (45). A study based on the environmental tolerances of WSPP1 and WSPP2 CMRSA strains, compared with multi-resistant hospital-acquired MRSA, found that the CMRSA's were more tolerant of high concentrations of salt. In all other aspects (skin fatty acids, UV light and desiccation), there were similar tolerances between the strains (18).

On looking at the physical attributes of MRSA and MSSA it seems that there is little difference between the two. MRSA may last significantly longer in the environment than MSSA; this demonstrates the need for good cleaning practices within health-care facilities. Some MRSA strains may be more able to cause infections than other MRSA strains (as is also the case with MSSA); as little published work has been done on the latter factor few conclusions can be made. *Staph aureus* seem to be no less virulent or contagious than before; if left with no intervention, outbreaks of both MRSA and MSSA happen readily. Hospitals experience outbreaks of both MSSA and MRSA infections, but because antibiotics can be used to treat the former, and containment and medical intervention can help with the latter, outbreaks can more easily be controlled. However, outbreaks of MRSA are more difficult to contain because of their inherent resistance to antibiotics, and so can cause major disruption to hospital services when infection control procedures are brought in to contain the spread of infection.

Hence, neither virulence nor transmissibility on their own can indicate why MRSA causes such problems. Perhaps there are factors present in the host that should be taken into account.

### Host characteristics

Bacteria invade the host. There are often particular things that are present in the host that promote infection, changing the status of an organism from a commensal to a pathogen, and in hospitals this is important.

As with many other infections, some people are more prone to getting MRSA infections, and for some these infections may be extremely serious. This could depend on the strain of organism or it could depend on other underlying conditions that people may have. Other people may carry the organism without it causing any infection at all, as part of their normal skin and nasal flora.

Some people have been found to carry MRSA as a commensal, and many studies have been done to find if there were any predictable factors that pointed to some being more susceptible than others. These studies found that the elderly person, more often men than women (contrary to the mouse experiments mentioned before), and with a past history of exposure to antibiotics, hospitals and nursing/community homes within the previous six months, were prone to MRSA infections or carriage (7,11,12,20,43,47-52). The exposure to hospital life as a risk factor is understandable as that is where many multi-resistant bacteria are found, and so these people may have unknowingly acquired MRSA on previous visits. Also at risk are insulin dependent diabetics, presumably because of their need to invade the skin barrier with their constant use of syringes, and their altered

physiology. Pressure sores, though not varicose ulcers, are also positive predictors of MRSA, indicating that those requiring intensive nursing are more at risk.

Some MRSA infections can progress on to produce bacteraemia, and it has been found that where bacteraemia originates from MRSA-associated pneumonia and abdominal infections, there is a more significant chance of morbidity than those where bacteraemia originated from other foci (50). There appears to be no logical explanation for this apart from the other underlying factors that may also be affecting these patients.

In people suffering from liver diseases, any strain of MRSA has been found to be more virulent than MSSA. Of these patients, those who had nasal carriage of MRSA have also been found to develop infections caused by MRSA sooner after liver transplant than with any other organism, and there was a significant risk factor for MRSA but not MSSA for subsequent infections, of which 56% progressed on to produce MRSA bacteraemia (50,51). This extra susceptibility is thought to be because of the effect of liver disease on granulocyte function.

It seems that some contribution to MRSA morbidity does come from the state of the host. An extra factor that must be taken into account when examining host characteristics is the use of antibiotics that comes with illness.

### Antibiotic use

The question of antibiotic use in general increasing the risk of carrying or being infected with MRSA is a curious one, as several studies have not found this to be the case (28,52). Of particular note was a matched case-controlled study carried out in a German hospital. When they looked at the different groups of antibiotics used, they found that only the fluoroquinolone group (e.g. ciprofloxacin) emerged as a significant independent risk even though it is not generally used for staph infections. It has been shown that clinical isolates of ciprofloxacin-resistant MRSA, on exposure to low concentrations of ciprofloxacin, will produce fibronectin-binding proteins. This increases their ability to adhere to fibronectin-coated surfaces and also to foreign-body implants in a guinea pig model. This has obvious implications for those people with such things as heart valve replacements. This type of antibiotic is also readily excreted in the sweat and sebum, achieving inhibitory concentrations of the drug on the skin surface and thereby altering the normal skin flora of healthy people. This means that there could be an increase in the level of MRSA skin colonisation in clinical settings where fluoroquinolones are often used, which could then lead onto more serious infections. With this in mind the authors of this study urged for prudent use of fluoroquinolones in hospitals where MRSA is endemic (28). Care is also needed when treating those with indwelling catheters and other fibronectin-coated products. In light of the recent anthrax infections in America and the prophylactic use of ciprofloxacin to protect those exposed to the bacillus, it will be interesting to see if the rate of MRSA infections changes at all.

It seems, from the above discussion, that no single factor can explain why MRSA can be so problematic within hospitals. When the human factor is added to the physical factors of the bacteria, a more complex picture emerges. On the one hand a healthy person can carry a strain, and it causes no harm; on the other, the same strain may be producing secondary pneumonia which may progress on to bacteraemia and then, perhaps, death. This is true of both MSSA and MRSA.

The biggest, and predominant, difference between MSSA and MRSA is that MRSA is resistant to all (-lactam antibiotics. This makes treatment difficult. Its ability to last outside the host longer than MSSA, and that factors relating to the host influence the outcome of infection

and carriage, mean that ways of keeping MRSA out of hospitals (or at least at manageable levels) have been developed.

### Hospital strategies

The task of keeping MRSA out of hospitals has become so enormous as to seem to be almost impossible and now many do have endemic MRSA (53). However, when MRSA accounts for >5-10% of clinical isolates, the use of glycopeptides to treat clinical MRSA infections may increase dramatically. The overuse of these antibiotics is a constant threat, both to the environment and to the health budget, so efforts to stop or contain MRSA outbreaks must continue (38). On the whole, early detection of MRSA-colonised patients can decrease the total number of days of MRSA isolation by 42% and, by preventing HAI, reduces the annual cost to the hospital by up to hundreds of thousands of dollars. The same study also postulated that if MRSA were prevented from being transmitted to as few as six patients, the intervention would be cost effective (51). Another stated that one MRSA-positive patient generated an extra twenty days in hospital, while another compared 51.4 days for those with MRSA to 32.2 days for those that did not, in a geriatric acute assessment and rehabilitation ward (7,43).

The technological age has given the hospital system the ability to identify known carriers of, and those at risk of carrying, MRSA, as soon as they enter the hospital. A number of reports on the use of computer early warning systems have indicated how useful these are with regard to selecting these people (51,54,55). The population to target on admission to hospital are those transferring from another hospital or care facility, past carriers of MRSA, and those exposed to a hospital, care facility or antibiotics within the last six months. A French study carried out in 1996-1997 showed that a selective screening strategy was as sensitive as the systematic screening of all incoming patients, and that 96% of incoming patients who carried MRSA would have been openly admitted onto a ward if they had not been screened at all (56). Also of concern are health-care workers, who may carry MRSA into a hospital. Pre-employment screening is of great value with this group of workers (15,17). There are many studies on ways of screening for MRSA and will not be discussed here (27,33,55,56).

When MRSA has been isolated, it is possible to keep MRSA transmission to a satisfactory level (57). One paper reviewing this problem reported on a US hospital that dropped their rate of HAI by 50% by using targeted screening of patients admitted from chronic care facilities, an isolation technique based on hand washing, and feedback to physicians. Hand washing and patient isolation also worked in another US hospital. In Hong Kong, after four years of trying to eradicate MRSA it was decided to contain MRSA by modifying the containment strategy, and they decreased the incidence rate from 1.14 to 0.52 per 100 discharges, and the rate of MRSA bacteraemia from 25% to 9.2%. The paper summarised by emphasising the use of realistic screening, improved hand hygiene practises and early implementation of patient isolation to manage endemic MRSA (51).

Extra time in hospital always costs money, so it seems that for the overall good of the patient and the hospital, screening practices should continue, and the simplest and most effective way to prevent cross infection of MRSA is by hand washing, whether by soap and water or alcohol hand rubs (7,12,28,51,58-60).

### Conclusions

The major and stable difference between MRSA and MSSA is the presence of the *mecA* gene. The only genetic difference found, so far, between hospital-acquired strains of MRSA and CMRSA is the shortened version of the SCCmec type 1V element and the PVL gene, both found only in CMRSA. The fact that the *mecA* gene can be horizontally transferred to an MSSA strain does cause concern as an

otherwise susceptible strain of *Staph aureus* then becomes resistant to  $\beta$ -lactam antibiotics, along with whatever antibiotics it was resistant to in the first place.

The fact that MRSA or MSSA can cause infections in individuals depends on what else is wrong with the patient, and the ability of the organism to change in order to survive in a changing environment. In today's global society the staphylococcus that will become the most problematic will be the one that is able to survive the last antibiotic invented, and that may not have anything to do with whether it can produce a more severe infection than its more sensitive brothers. Once any staphylococcus is allowed to flourish it seems to be able to take advantage of any situation. The question of if (and why) one strain of MRSA is more contagious than another remains unsolved, probably because there are so many variables to include in the argument. In addition, the finding that the fluoroquinolone group of antibiotics may promote MRSA carriage is important; research is needed to confirm this finding so that its significance can be fully understood.

It can be seen from this discussion and epidemiological studies just how easily MRSA can spread throughout hospitals. Given the increased cost of treating and caring for infected patients, and compared to the success of vigilant nursing practices and screening methods, MRSA can and must be kept out of hospitals.

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# Faecal occult blood testing: guaiac vs immunochemistry: which method should we use ?

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## Abstract

The faecal occult blood test has evolved as a screening test for colorectal cancer. Historically Guaiac-based testing has been used for this purpose. However immunochemical detection methods have become increasingly recognised as a more cost effective and specific test when screening for colorectal cancer. Results from an external quality control program, a survey of clinicians, a small parallel study and trends in other countries all show a preference toward immunochemistry.

**Key words:** colorectal cancer, occult blood, guaiac-based, immunochemical, gastrointestinal bleeding, specificity

## Introduction

Colorectal cancer is the second leading cause of cancer death in many developed countries. There are many methods available for screening patients for colorectal cancer. We have reviewed the background to this and the strengths and weaknesses of two common, yet different faecal occult blood screening methodologies employed by various laboratories throughout New Zealand. A screening test should be sensitive, specific to the disease and cost effective.

### Occult blood detection - history

In 1901, Boas originally suggested the value of occult blood for the detection of bowel malignancy (1), although it wasn't until the mid-1960s when Greegor reported the detection of several asymptomatic tumours (using guaiac-impregnated slides) that clinicians became more interested (2). Many screening studies have subsequently been reported.

The concept of occult blood detection is credited to Van Deen, who in 1864 used gum guaiac as an indicator reagent (3). The terms guaiac acid screen and occult blood screen became synonymous and even today this is probably the most widely used indicator for gastrointestinal (GI) bleeding. In the early 1900s, Alder and Alder introduced benzidine as an alternative marker, and other technical variations followed (4).

Numerous other chemicals have been proposed to detect faecal blood, of which only guaiac, benzidine and orthotolidine have ever achieved any measure of acceptance. Since the 1960s, the later two have fallen into disfavour, partly due to concern about their potential carcinogenicity, but mainly because they are too sensitive for clinical validity. More recently, other methods have been developed including tests which detect stool transferrin, albumin, and mutations in the p53 and Ras gene.

Faecal occult blood is not considered to be an appropriate test for the detection of upper GI tract bleeding ([www.rcpamanual.edu.au](http://www.rcpamanual.edu.au)), even though some clinicians may continue to regard it as such. It is intended as a more specific screening test for lower GI tract bleeding, and in particular for colorectal cancer. This has implications for the optimal choice of method, which are discussed below.

### Where does the blood come from?

Bleeding may occur anywhere from the mouth to the anus and may be overt or occult (hidden). Vomiting of blood (haematemesis), passage of black tarry stool (melena) and passage of gross blood per rectum (haematochezia) have all been regarded as valid reasons for occult blood testing.

Haematemesis or "coffee ground" emesis indicates an upper GI bleed, with the colour resulting from bleeding that has slowed or stopped followed by the conversion of red haemoglobin to brown haematin by gastric acid. Bright red vomitus indicates active bleeding.

Haematochezia usually indicates lower GI bleeding, but may result from vigorous upper GI bleeding with rapid transit of blood through the intestines. It is usually an indicator of distal lesions e.g. haemorrhoids, inflammatory bowel disease, cancer or polyps.

Melena typically indicates upper GI bleeding, but a small bowel or right colon bleeding source can present with melena. About 100 to 200 ml of blood in the upper GI tract are required to produce melena (5). Melena may persist for several days after a severe haemorrhage and does not necessarily indicate continued bleeding. Other causes of a black stool (not necessarily associated with blood loss) include ingestion of iron, bismuth and a variety of foods.

### The faecal occult blood test (FOBT)

Faecal occult blood testing is based on the premise that polyps and cancerous lesions bleed more than normal mucosa. The amount of bleeding is related to the site of the cancer (highest blood loss from large lesions in the caecum and ascending colon) and increases with size of the polyps and the stage of the cancer. This bleeding is also intermittent and unevenly distributed in the stool, necessitating multiple sampling. The generally accepted collection protocol is three samples obtained on different days.

An investigation of samples sent to Canterbury Health Laboratories (mainly hospital based patients) showed that only 22% of patients presented the lab with the suggested three samples. Another laboratory serving mainly a community based patient population found that 79% of patients present a complete set of three samples. Either hospital based patients are discharged before they complete the three samples or else there is a lack of understanding regarding how the test should be done correctly. It is also possible that the FOBT test is being used as a one-off test for generalised bleeding, although as indicated above this is not an appropriate use of FOBT.

### Guaiac-based tests.

The pseudoperoxidase activity of haem, either as intact haemoglobin or free haem, converts colourless guaiac to a blue colour. While most convenient and practical, due to its nature, this chemical reaction is neither specific for blood nor does it give any indication of the amount of blood detected. In order to minimise false positives, patients should avoid aspirin and non-steroidal anti-inflammatory drugs for one week (to prevent potential upper GI bleeding). Advice should also be given to avoid eating red meat, raw fruits and some vegetables (especially



melons, cauliflower, parsnips, radishes, turnips, horseradish and broccoli) to minimise confounding sources of peroxidase. Vitamin C, an antioxidant, may produce false negative results if intake exceeds 250 mg/day. Some manufactures also suggest that iron supplementation can interfere with their method, due to its oxidative effect, although this has been disputed by several authors (6).

These dietary and medication restrictions, if taken into consideration, would render this test redundant in most acute hospital settings. Even in most hospital wards or general practices few doctors take into consideration what the patient has just eaten when ordering a faecal occult blood test. The majority of samples received by the laboratory would probably not have had these restrictions adhered to. In the event of a positive result, this leaves the follow-up specialist the option of either repeating the occult blood collections or proceeding to colonoscopy.

### **Immunochemical detection methods**

These include monoclonal and/or polyclonal anti-human haemoglobin antibodies that react with the globin portion of non degraded haemoglobin. If haemoglobin is present in the stool, the labelled antibody recognises its antigen, giving a positive test result.

Haemoglobin from upper GI sources is generally degraded by bacterial and digestive enzymes before reaching the large intestine and is therefore rendered immunochemically non-reactive, unless there is either a large amount of blood or fast passage of the blood through the upper GI tract. Haemoglobin from lower GI sources, on the other hand, undergoes less degradation and therefore remains immunochemically reactive.

This method may thus offer improved specificity for lower GI lesions with associated bleeding (7). Furthermore, because it does not cross react with myoglobin or haemoglobin from non human origin and is not affected by either vitamin C, iron or plant peroxidases, the number of false positives and false negative results should be much lower. The lack of dietary restrictions may also enhance patient compliance.

In some immunochemical methods, sample collection is performed by swirling a long-handled brush in the toilet water containing the stool thus trapping dislodged occult blood and water on the bristles. The brush is then dabbed onto the test card where the water dries into a special pad. Once dry, the haemoglobin is considered stable and the card can be sent to the laboratory.

The American Cancer Society has recently made recommendations for screening and early detection of adenomatous polyps and colorectal cancer and stated that "in comparison with guaiac-based tests, immunochemical tests are more patient-friendly, and are likely to be equal or better in sensitivity and specificity" (8).

### **Does FOBT testing improve clinical outcomes?**

A systematic review included a meta-analysis of four randomised and two non-randomised trials of Hemoccult (guaiac based) screening in five countries (9). This showed a reduction in total mortality of 16% in those allocated to screening (relative risk: 0.84; 95% confidence interval: 0.77-0.93). Further analysis suggested that if a biennial Hemoccult screening programme were offered to 10,000 people (and about two thirds attended for at least one test), 8.5 deaths from colorectal cancer would be prevented over 10 years (9). However, it needs to be remembered that in the major trials using guaiac based methods, over 80% of positive results have been falsely positive and have required invasive and expensive investigation, including colonoscopy (9). In one of the trials included in the meta-analysis, the Minnesota trial (10), there was an overall 19% reduction in the risk of death with screening (relative risk: 0.81; 95% confidence interval 0.65-

1.02). There were four cases of perforation and 11 cases of serious bleeding resulting from 12,246 colonoscopies (12 complications per 10,000 colonoscopies). The systematic review concluded that more information is needed about the harmful effects of screening and its attendant costs before widespread screening can be recommended (9).

Although immunochemical methods in general are 3-4 times more expensive than guaiac based ones, the potential for fewer false-positive screening outcomes and less unnecessary and costly investigation should impact favourably on any cost-benefit analysis. This may, at least in part underlie the decision of an ongoing Australian pilot screening programme to adopt immunochemical methods.

### **External quality assurance schemes**

We are only aware of one such scheme, namely the Yorkshire Quality Assessment Scheme (YQAS). This scheme is run on the premise that the cut-of for positive/negative is 1.0 ml blood per 100 g faeces, and with the understanding that the scheme is designed for the quality assurance of screening for lower GI tract (colorectal) cancer.

This scheme distributes one sample per week containing various amounts of blood (ranging between 0 and 2 ml of whole blood per 100 g of matrix), and vitamin C or iron. Participants are asked to submit results as negative/trace/positive. Results are received in the form of a spreadsheet showing the range of laboratory method results and the amount of material added to the imitation faecal matrix.

This program, however, does have its limitations in that it is only useful for Guaiac based methods as the sample preparation procedure degrades the blood to the extent that it is no longer immunochemically active.

Overall results indicate that samples fortified with iron have no effect on results. Sodium ascorbate does significantly affect results (false negatives) in concentrations of 10 mg/100g of matrix or higher.

It is also apparent that for all the methods used by participants (7 methods over 64 laboratories), the threshold for a positive result (0.4 - 0.5 ml whole blood per 100g matrix) is actually at a much lower level than that set by the National (English) steering committee guidelines. There are, thus, an unacceptably high number of false-positives which have brought into question the value of FOBT testing.

The YQAS annual report 2003 states that "discussions about the diagnostic confusion that may result from using occult blood testing have led to consensus amongst lead clinicians in Chemical Pathology, Haematology, Gastroenterology and Colorectal Surgery that the test should not be used for diagnostic purposes. Several consultants were in favour of abandoning occult blood testing altogether in our trust." The real question, however should be rather directed at the choice of method rather than FOBT testing per se.

### **What's happening in other countries?**

In Australia, the UK, and the United States, colorectal cancer is the commonest cancer in women after breast cancer (age standardised incidence: 22-33 per 100,000) and in men after prostate and lung cancer (age standardised incidence: 31-47 per 100,000) (11,12). Unfortunately, fewer than 40% of these cancers are detected at a stage when they may be more easily treated. The Australian government has taken the initiative and set up a pilot study to address this high incidence of undetected bowel cancer. This is a study that will examine the feasibility, acceptability and cost effectiveness of FOBT testing throughout Australia. This pilot study has elected to use two immunochemical methods as the first line screening test, presumably recognising that guaiac based tests are not sufficiently specific.

### **How much difference is there?**

A small parallel study reveals somewhat surprising data. Twenty

seven routine patient samples received for FOBT testing at Canterbury Health Laboratories were analysed by both guaiac and immunochemical methods. The guaiac method gave 10 positive results while the immunochemical method gave 4 positive results. No samples were negative by guaiac but positive by immunochemistry. Therefore six samples were positive by guaiac and negative by immunochemistry. Are these false positives for the guaiac method or are they false negative by the immunochemistry? Further investigation of these six samples found that two samples were on the same patient; one patient had been referred on to a specialist based on the single positive result, it was unclear where the bleeding was and why the patient was investigated for FOBT. The remaining patients clearly (based on clinical investigations) had bleeds from upper GI sites and were not being investigated for colon cancer. It appeared that the FOBT testing was used erroneously to support an already clear diagnosis.

#### **So what are clinicians using the FOBT test for? Do they have a preference on which type of test?**

A survey of several clinical groups generated very few responses. The options were: continuing with the guaiac approach, switching to an exclusively immunochemical approach (more expensive but more specific), or a two-tiered approach of an initial guaiac screen followed by immunochemical "confirmation". One reply was "we want the best test", interpreted as: you the laboratory decide! The only decisive comment came from a group of gastroenterologists who preferred the immunochemical test exclusively.

## **Conclusions**

Laboratory medicine is an evolving discipline. Similarly, a laboratory test may also change or it may have different expectations imposed on it by the clinicians that use the test. The FOBT test is a good example of this.

It is the responsibility of the laboratory to offer the best test for any condition. Groups such as the YQAS have recognised that the test expectations have changed, but have not realised that the guaiac based tests cannot actually change. A positive is still a positive regardless of preset criteria. If Gastroenterologists, the very group expected to follow-up on all the positive FOBT tests, prefer an immunochemical approach, then the value of the guaiac acid test needs to be questioned. Even though guaiac tests are cheaper to perform, the cost of follow-up investigations for the false positives may exceed the projected laboratory savings.

Some laboratories around the country have already changed, others are in the process of changing, yet others are reticent to change. The immunochemical approach is more expensive, though it confers improved specificity. Ongoing trials may help to quantify the cost-benefit advantage of an immunochemical approach.

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## Minutes of the 23rd AGM of the Pacific Paramedical Training Centre, 7 May 2004

### Present

Dr Ron Mackenzie	Chairman
Mr Mike Lynch	Treasurer
Ms Marilyn Eales	NZIMLS
Mr Rob Siebers	Wellington School of Medicine
Ms Clare Murphy	Wellington Hospital
Ms Christine Story	PPTC
Mr A Simes	Accountant
Ms Pat Maddocks	Wellington Hospital
Ms Angela Brounts	Wellington Hospital
Ms Maureen Cahill	Vic Link, Wellington
Mr Malcolm	Vic Link, Wellington
Mr John Elliot	PPTC

### Apologies

Dr Sandy Ford	
Dr Russell Cooke	Wellington Hospital
Mr Andrew Crooke	Wellington Hospital
Mr Stewart Dixon	
Ms Ruth Holland	NZAID
Ms Vanessa Thompson	Hawkes Bay Regional Hospital
Mr Tirath Laksman	Hawkes Bay Regional Hospital
Mr Murray McDowell	Technology & Training Transfer
Ms Carolyn Graham	NZ Blood Service

### 1 Apologies:

The Chairman asked that the apologies be received. The motion to receive apologies was moved by Mike Lynch, seconded Clare Murphy. Carried.

### 2 Minutes:

The Minutes of the 22nd AGM had been circulated. There were no alterations or corrections. Marilyn Eales moved and Clare Murphy seconded, "That the minutes of the 22nd Annual General Meeting of the PPTC be approved as circulated." Carried

### 3 Matters arising:

#### 3.1 Expenditure

The chairman reported that a programme to replace and update some of the Centre's equipment was under way and would be continued in 2004.

#### 3.2 PNG Training Programme

There has been no further contact with the authorities in PNG although John Elliot reported that he discussed the matter with the PNG, WHO Microbiology scientist while at a WHO Meeting in Manila in October 2003 and it is hoped that progress can be made during 2004.

#### 3.3 Bhutan

Following Clare Murphy's most recent visit to Bhutan, the laboratory, in Thimphu has joined the REQA Programme, participating in the biochemistry, microbiology, serology and blood bank surveys.

### 4 Annual report

The Annual Report was distributed at the commencement of the meeting and the Chairman spoke to it highlighting the various activities of the Centre during the past 12 months. He indicated that matters relating to the programme would be discussed as a separate agenda item. After brief discussion it was moved Ron Mackenzie, seconded Rob Siebers that, "The 2003 Annual Report of the PPTC be accepted." Carried.

### 5 Financial report

The Financial Report was distributed at the commencement of the meeting and the Treasurer, Mike Lynch, and the accountant Mr A. Simes spoke to it highlighting the following:

#### 5.1 WHO fellowship programme management

Vic Link now has the contract for managing WHO Fellowship students coming to PPTC courses and hence the financial impact on the PPTC from 2002 has now been reversed with Vic Link now handling all the finances associated with this programme.

**5.2 Income:** The total income for the year was down compared to 2002 due to the situation detailed above and also as there was only one course held at the Centre during the year. The Treasurer advised that WPRO had agreed that they would again have a financial contract with the PPTC for the provision of the REQA Programme in 2004.

**5.3 Expenditure:** In the majority of categories the expenditure for 2003 was similar to that for 2002 except for those areas directly associated with courses where the expenditure was considerably reduced again to Vic Link taking over management of the WHO Fellowship Programme.

**5.4** A note of warning was made with the expenditure for 2003 exceeding the income by \$20,674, saying that further avenues of income may need to be sought in the near future.

It was moved Mike Lynch, seconded Marilyn Eales that: "The Financial Report of the PPTC for 2003 be accepted." Carried.

## **6 Election of Committee and Chairman:**

The Chairman advised that all members of the Committee were willing to stand for re-election; he then called for further nominations. As there were none it was moved Clare Murphy and seconded Angela Brounts that the current committee members be re-appointed for 2004. This was carried.

The Chairman stated that he was willing to stand for a further year. Further nominations for Chairman were called for but as there were none it was moved Mike Lynch and seconded Rob Siebers that Ron Mackenzie be re-elected Chairman of the PPTC for 2004. Carried

## **7. Programme for 2004**

**7.1** The programme for 2004 was included in the Annual Report and it was not envisaged that there would be any changes to this.

### **7.2 AFB QA programme**

John Elliot attended the 2nd Stop TB Meeting in Noumea, organised by WHO and SPC in March. As a result of this the AFB QA Programme will continue and the PPTC will be responsible for the programme in the Polynesia Group of countries.

### **7.3 Distance learning**

In December the PPTC was approached by WHO to prepare a pilot distance learning course for the Pacific Open Health Learning Net [POHLN] run by WHO in Suva. We agreed and signed an APW to prepare a course on The Laboratory Diagnosis of Bacterial Diarrhoeal Diseases. It is hoped that this course will be the first of a continuing series.

**7.4** There was some discussion regarding the advantages and disadvantages of conducting courses at the PPTC as compared to "in-country" and/or distance learning. The general consensus was that all three have a place.

## **8. General**

**8.1** Laboratory space: John Elliot reported that during the past 12 months the Pathology Department in the Wellington School of Medicine have redeveloped their student laboratory area to meet their requirements for microscopy use only. This means that this laboratory is now not suitable for Microbiology courses run by us and we will need to find alternative laboratory space if we have more than 5 students on a course.

It was suggested that other areas in the School of Medicine, ESR Gracefield, and Massey University be contacted regarding the availability of laboratory space.

**8.2** Charities bill: Mike Lynch reported that the government was in the process of introducing a bill to control the activities of charitable organisations. It was agreed that we keep a watching brief on these developments but that we would not make a submission ourselves as CID is making a submission on behalf of organisations in the development sector.

**8.3 NZIMLS:** Rob Siebers reported that the NZIMLS Council at its recent meeting had approved the donation of \$5,000 to the PPTC. He also reported that the article on the PPTC REQA Programme published in a recent edition of the NZIMLS Journal had drawn considerable favourable comment at the meeting.

**8.4 Donations:** After some discussion on the reporting of donations in the Annual Financial Report, Rob Siebers moved and Marilyn Eales seconded that: "Income from donations made by both individuals and organisations be noted separately in the Financial Report." Carried.

There being no further business the meeting concluded at 3.00pm.


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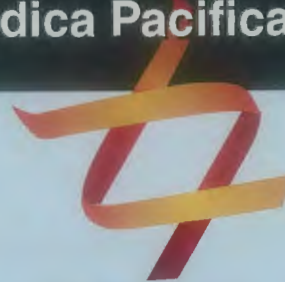
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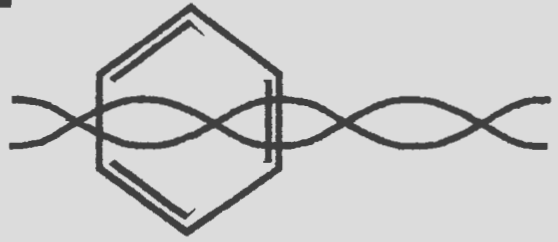


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# Biochemistry

## Special Interest Group



### **Biochemistry Special Interest group meeting. April 3rd 2004, Heritage Hotel, Auckland.**

The seminar was held this year with the resurrected Occupational Health and Safety SIG meeting. The opening plenary on stress management presented by Dr Jim McLeod placed everyone in the right frame of mind for the rest of the day. His presentation style delivered an underlining message that had a huge relevance for most of the audience.

The two groups split into their respective disciplines for the rest of the day. The papers presented within the Biochemistry forum were both interesting and varied. Dr Mark Harris, a paediatric endocrinologist at Starship and the Liggins Institute, spoke on the current thinking the role of leptin, adiponectin and ghrelin have in biochemical regulation. Merle Sheat from Canterbury Health Labs did an excellent presentation on an unusual case of Monoclonal Gammopathy, and Ron Couch from LabPLUS followed with a dissertation on benzylpiperazine, a new designer drug in New Zealand.

The prize for the best Biochemistry paper was awarded to Chris Sies, Canterbury Health Labs for his paper on Azathioprine, a medication that could be the death of you. Abbott Diagnostics were the sponsor of the best paper and must be thanked for their generosity. An interesting paper on iodine contamination in patients attending thyroid clinic was presented by Roger Johnson of LabPLUS concluded the morning session.

Straight after lunch Karen Snow-Bailey, the new director of Diagnostic Genetics at LabPLUS, presented on the new area of proteomics and the opportunities for the biochemists. Trevor Walmsley, convener of the Biochemistry SIG from Canterbury Health Labs

presented mass spectral analysis; application of mass spectral deconvolution to the analysis of organic acids in urine. Blood gas POCT analysis came under the scrutiny of Craig Rouse from LabPLUS when presenting the dilemma of whole blood sodium analysis on the blood gas analyser compared with the serum analysis of sodium in the core lab. ISO 15189 accreditation is a subject dear to all biochemists and laboratories hearts at the moment. A forum discussion lead by Kristen Kelly of LabPLUS on this subject finished the first of the afternoon's sessions. The effect of pseudoephedrine on the CEDIA quantitative amphetamine assay was the subject of Capital Coast Health's Angela Brounts.

The functionality of Auckland City Hospitals new Admission and Planning Unit was the subject of its clinical director's presentation. Dr John Henley went on to explain the pivotal role the laboratory had in this "Hospital within a Hospital" with his own role as a senior consultant described as the "grunt in the front". This very amusing and fascinating paper connecting the laboratory's role with patient outcomes and management concluded the Biochemistry section of the seminar. The final presentation from the NZIMLS explaining its role and the CPD programme to the combined groups completed the formal programme. Later, pre-dinner drinks and a BBQ buffet rounded off what was a very successful day. Copies of all the presentations have been posted on the NZIMLS website [www.nzimls.org.nz](http://www.nzimls.org.nz)

We would like to thank all the participants, delegates and the sponsors, Abbott Diagnostics, Roche Diagnostics, Bayer Healthcare, Radiometer Copenhagen, Dade-Behring, Beckman Coulter, and LabPLUS.

*Ross Hewett*



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