This was a retrospective descriptive study using stored DNA extracts from respiratory specimens that tested positive for *M. pneumoniae* using a real-time PCR assay, targeting an 89bp fragment of the repMp1 gene of *M. pneumoniae*, at the Auckland City Hospital laboratory, Auckland, New Zealand, between September 2016 and April 2020. Cases were identified from laboratory records and basic demographic and epidemiological data were extracted from electronic health records. Only cases from persons in the Auckland region were included. The presence of macrolide resistance associated mutations was assessed by PCR amplification of the peptidyltransferase (domain V) region of the 23S rRNA gene using primers described by Wolff *et al.* (5′-3, forward AACTATAACGGTCCTAAGGTAGCG; reverse GCTCCTACCTATTCTCTACATGAT) followed by sequencing of the amplicon using an Applied Biosystems 3130x sequencer.[7] Sequences were aligned against *M. pneumoniae* M129 reference genome (NCBI accession number NC_000912.1) using SeqMan II bioinformatic software (DNAStar, USA), with 23S rRNA mutations described using *M. pneumoniae* numbering. Study approval was obtained from the New Zealand Health and Disability Commission Ethics Committee (reference number 21/CEN/100). Funding was provided by the A+ Trust Microbiology Education and Research fund.

Ninety *M. pneumoniae* cases from the Auckland region were identified for possible inclusion in this study. Seventy-nine of these cases had stored DNA extracts available for further testing; these included 39 from 2016−2018 and 40 from 2019−2020. These 79 cases had a median age of 22 years (range 0.5−92.5) and 42 (53%) were female. In 74 (94%) of the cases the specimen was collected in the inpatient or emergency department setting. Mutations in the 23S rRNA gene were detected in seven (9%) of 79 strains. All seven (100%) had the single-nucleotide polymorphism A2063G. One (3%) of 39 strains from 2016−2018 had a resistance associated mutation compared to six (15%) of 40 from 2019−2020. Four (57%) of the seven resistant strains were in persons ≤15 years of age. Six (86%) of the seven were in cases tested in the inpatient or emergency department setting.

This study is the first to describe the occurrence of macrolide resistant *M. pneumoniae* in New Zealand, with the common resistance associated mutation A2063G identified in 9% of strains evaluated, comparable to rates of resistance that have been reported in Europe and the United States.[1,2,4] As this study was geographically restricted to the Auckland region and consists primarily of hospitalised cases, the rate of resistance may not be generalisable across the country or to the infected population as a whole. Nevertheless, it illustrates the presence of macrolide resistant *M. pneumoniae* in New Zealand, the expanding global distribution of macrolide resistance, and the local need to consider the role of resistance and alternative antimicrobials in cases responding poorly to empiric macrolide therapy.

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Interference of methylene blue therapy on methaemoglobin measurement

To the Editor,
Methylene blue (MB) is a classical antidote for treating methaemoglobinaemia. Conventional co-oximetry utilises multi-wavelength spectrophotometer for methaemoglobin (MetHb) determination, which shares a similar absorption spectrum to MB.[1] Previous studies demonstrated significant analytical interference of MB on MetHb measurement, but the studies were conducted more than 20 years ago, and in one study the MB concentrations investigated were at supra-therapeutic range.[2,3] With the emergence of newer co-oximetry models and better pharmacokinetic profiling of MB, this study aims to re-investigate the effect of MB interference on MetHb measurement, by spiking clinically relevant MB concentrations to heparinised whole blood samples at varying MetHb concentrations.

In the current study, MetHb concentration in heparinised whole blood sample was measured with ABL800 FLEX co-oximetry (Radiometer, Denmark), which utilised a 128-wavelength spectrophotometer, at a measurement range of 478−672 nm.[4] The analytical coefficient of variation (CV_A) of the two co-oximetry analysers used in this study was estimated to be 1.6−2.1% at MetHb concentrations of 11.5−28.5% unit.

MetHb samples were prepared in-house by treating heparinised whole blood samples with sodium nitrite solution as described by Shihana *et al.*[5] and Lim *et al.*[6] In brief, sodium nitrite solutions were prepared by dissolving sodium nitrite standard (BDH, Prod 102564W, Lot A608266 617; VWR International Ltd, USA) in normal saline (0.9% sodium chloride) to a final concentration of 100, 500 and 2000 mg/L. Two hundred µL of one of these sodium nitrite solutions was added to 1 mL of heparinised whole blood sample collected from healthy volunteers. The spiked blood samples were mixed and incubated for 5−10 min at room temperature before

subjecting to MetHb measurement. Blood samples with MetHb concentrations from approximately 10%−60% unit were prepared.

MB stock solution of 10 mg/mL (DIN:02230770, Lot L0010072; Omega, Canada) was diluted with normal saline to obtain working solutions at final concentrations of 10, 100 and 400 mg/L. Sixty μL of one of these working MB solutions was added to 1.2 mL in-house prepared MetHb blood samples to reach final MB concentrations of 0.5, 5 and 20 mg/L. Control aliquots were prepared for each data point, by spiking 60 μL normal saline without MB. Each sample was compared with its own control and MetHb measurement was performed immediately after treatment of the blood sample with the spike. The absolute difference (% unit) and relative difference (%) in MetHb measurement between the MB-spiked sample and its corresponding control were calculated as below.

Absolute difference in MetHb concentration (% unit) = MetHb concentration in MB-spiked sample (% unit) − MetHb concentration in corresponding control sample (% unit).

Relative difference in MetHb concentration (%) = Absolute difference in MetHb concentration (% unit) ÷ MetHb concentration in control sample (% unit) × 100%.

With reference to the Analytical Performance Specifications of the Royal College of Pathologists of Australasia Quality Assurance Programs and our in-house CV_A on MetHb measurement, we define a significant difference in MetHb concentrations measured between the spiked and control samples as ±2.0% unit (absolute difference) at MetHb concentrations less than or equal to 20% unit, and ±10% (relative difference) at MetHb concentrations greater than 20% unit.[7]

Results for MetHb concentrations measured in the MB spiked samples and the corresponding control samples were listed in Table 1.

Previous studies by Gourlain *et al.*[2] and Dötsch *et al.*,[3] published before the millennium, demonstrated that MB posed a significant negative interference on MetHb measurement with a conventional ABL co-oximetry (ABL520 model, utilising 6 wavelengths for measurement). In Gourlain's study, the effect of spiking MB, at concentrations of 10−100 mg/L, into blood samples with MetHb concentrations of 4.6−14.5% unit was evaluated. The MetHb concentrations measured in the MB-spiked samples showed an absolute difference of −3.1% to −34% unit, when compared to the corresponding unspiked samples. However, these data may not be readily applicable to our current laboratory practice, as the modern co-oximetry models utilise more wavelengths for measurement and are theoretically less susceptible to spectroscopic interference by MB (ABL800 FLEX, utilising 128 wavelengths for measurement). Moreover, with improved understanding of MB pharmacokinetic profiling, MB spiked in the above study were actually at supra-therapeutic concentrations.[8] Furthermore, the manufacturer manual of the co-oximetry used in the current study only provided limited data concerning the potential interference of MB on MetHb measurement. According to the manufacturer's claim based on mathematical superimposition of interference spectra and calculations, MB at 30 mg/L was predicted to cause a negative interference in MetHb measurement with an absolute difference of −6.2% unit in a sample with 0.5% unit MetHb.[4] Still these data are not clinically useful, as MB interference was only estimated at a single supra-therapeutic concentration at which the MetHb level was clinically insignificant.

Table 1 MetHb concentrations measured in blood samples with or without MB spiking

	MetHb concentration(s) measured (% unit)		Absolute difference (% unit)[a]	Relative difference (%)[b]
	Sample with MB spiked	Control sample with no MB spiking		
MB spiked at 0.5 mg/L				
MetHb level 1	13.9	13.7	+0.2	+1.5
MetHb level 2	36.3	36.0	+0.3	+0.8
MetHb level 3	46.1	46.6	−0.5	−1.1
MetHb level 4	61.9	63.3	−1.4	−2.2
MB spiked at 5 mg/L				
MetHb level 1	10.7	14.7	−4.0	−27.2
MetHb level 2	37.3	38.7	−1.4	−3.6
MetHb level 3	39.5	39.3	+0.2	+0.5
MetHb level 4	50.4	52.9	−2.5	−4.7
MB spiked at 20 mg/L				
MetHb level 1	Interfere[c]	13.3	−	−
MetHb level 2	19.3	33.5	−14.2	−42.4
MetHb level 3	53.4	53.1	+0.3	+0.6
MetHb level 4	65.3	66.8	−1.5	−2.2

[a] Absolute difference in MetHb concentration (% unit) = MetHb concentration in MB-spiked sample (% unit) − MetHb concentration in corresponding control sample (% unit).
[b] Relative difference in MetHb concentration (%) = Absolute difference in MetHb concentration (% unit) ÷ MetHb concentration in control sample (% unit) × 100%.
[c] Error tag was shown on this measurement, and the MetHb concentration in this sample could not be measured.

In this study, the magnitude of MB interference at therapeutic concentrations on MetHb measurement was investigated. The conventional MB dosage for treating methaemoglobinaemia was 1−2 mg/kg given intravenously. Pharmacokinetic data showed that whole blood MB level reached a peak concentration of 3.2 mg/L, 5 min after intravenous administration of 100 mg MB, and eventually dropped to 0.3 mg/L at 30 min post-administration.[8] Therefore, MB concentrations of 0.5 mg/L and 5 mg/L were chosen for this study to mimic the blood concentrations after a therapeutic dose of MB. The study was also performed at a higher concentration of MB (20 mg/L) to replicate findings from previous interference studies by Gourlain *et al.*[2] and Dötsch *et al.*[3] The concentrations of MetHb chosen in this study were based on the relationship between MetHb concentrations, clinical manifestations and treatment threshold. Patients typically start to present with cyanosis at MetHb concentration of 10−20% unit; MB treatment is conventionally started in symptomatic patients with MetHb concentration >20% unit, or asymptomatic patients with MetHb >30% unit; and MetHb concentration >70% unit is usually fatal.[9] Therefore, we evaluated the effect of MB on samples with MetHb concentrations at 10−60% unit, which was the clinically significant range. In our study, MetHb measurement was made immediately after the addition of MB. This helped minimise the time for chemical reduction of MetHb, thereby allowing the investigation of pure physical interferences. The calculated difference in MetHb concentration between the sample and control set-up

therefore was attributed solely to the spectroscopic interference present.

At MB concentration of 0.5 mg/L, mimicking the typical therapeutic MB concentration 30 min after antidote administration, no significant interference was observed. Given the onset time for MB's action of 30 min,[10] it could be safely concluded that the interference on MetHb measurement would be minimal if blood taking is arranged 30 min after giving the MB antidote. In clinical practice, after MB treatment, patients with methaemoglobinaemia are monitored for resolution of symptoms and with serial MetHb measurements.[11] Repeated blood testing for MetHb is usually made in 1−3 h.[10,12] The results from our study are therefore in-line with the current practice, and could help reassure clinicians on the accuracy of MetHb concentration measured.

At MB concentration of 5 mg/L, which simulated the maximal MB concentration at 5 min after antidote administration, MetHb concentrations at >20% (levels 2, 3 and 4) were relatively unaffected. In contrast, a significant negative interference (absolute difference of −4.0% unit, equivalent to 27.2% relative decrease from the control aliquot level) was observed when MetHb concentration was <20% unit (level 1). Therefore, clinicians should be advised not to recheck MetHb concentration immediately after MB administration, as this may misguide clinical judgement and any subsequent management plan.

At MB concentration of 20 mg/L, a significant negative interference (absolute difference of −14.2% unit, equivalent to 42.4% relative decrease from the control aliquot level) was observed at lower MetHb concentration at approximately 30% unit (levels 2). At level 1 with MetHb concentration of approximately 10%, MB spiking even introduced an error signal and the MetHb level in this spiked sample could not be determined. Higher MetHb concentrations were relatively free from MB interference (levels 3 and 4). These data were consistent with the findings by Gourlain *et al.*[2] and Dötsch *et al.*[3] Modern co-oximetry, despite the improved technical specifications, is still subjected to MB interference at a very high concentration. However, this phenomenon is unlikely clinically significant as such MB concentration is much higher than the usual therapeutic range.

Several limitations existed in the current study. First, our interference study was carried out on the ABL800 FLEX co-oximetry only. It may not be possible to generalise our findings to other analysers. For laboratories using other models of co-oximetry it may be worth performing independent studies to evaluate the susceptibility of their analysers to MB interference. Second, the MetHb concentrations in the blood samples were not equally spaced, and they differed slightly between spiking set-ups. This could be attributed to the minor variations in incubation time and temperature. Third, in order to perform simultaneous MetHb measurement on both the spiked and its corresponding control samples, two independent blood gas analysers were employed, which may have introduced a larger CV_A.

In conclusion, an interference study of varyingly spiked MB concentrations on measurement of MetHb at 10−60% unit using ABL800 FLEX was performed. No interference was observed on MetHb measurement at the usual therapeutic concentration of 0.5 mg/L MB. A significant negative interference could be observed on MetHb measurement when MB was spiked at 5 mg/L, simulating the peak concentration after an intravenous dose. Clinicians are advised not to recheck MetHb concentration immediately after MB administration. However, they can be reassured that the measured MetHb concentration is not subjected to any significant MB interference 30 min after intravenous MB administration.

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A lesson learnt from laboratory error: isoelectric focusing improves the accuracy in interpreting polyclonal or monoclonal IgG4

To the Editor,
IgG4-related disease (IgG4-RD) is an immune-mediated condition characterised by fibroinflammatory lesions and