Chapter 74
Metallo β Lactamases Gene bla_{imp}, bla_{spm} and bla_{vim} Detection by Multiplex Real-Time TaqMan Assay on the Smartcycler

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74.1 Introduction

Gram-negative bacteria have a range of resistance mechanisms that they can use to evade the actions of carbapenems and other beta-lactams. The common form of resistance is either through lack of drug penetration (i.e., outer membrane protein (OMP) mutations and efflux pumps), hyperproduction of an AmpC-type beta-lactamase, and/or carbapenem-hydrolyzing beta-lactamases. Based on molecular studies, two types of carbapenem-hydrolyzing enzymes have been described: serine enzymes possessing a serine moiety at the active site, and metallo-β-lactamases (MBLs), requiring divalent cations, usually zinc, as metal cofactors for enzyme activity. MBLs, like all β-lactamases, can be divided into those that are normally chromosomally mediated and those that are encoded by transferable genes. The early studies on chromosomally mediated MBLs mainly centered around *Bacillus cereus* (BC II), and *Stenotrophomonas maltophilia* (L1). However, primarily due to genomic sequencing, increasingly more chromosomally mediated genes are being discovered but are often found across a range of bacterial genera.

Over the last decade there have been several articles summarizing the levels of MBLs in bacteria. However, in the past 3–4 years many new transferable types of MBLs have been studied and appear to have rapidly spread [3, 4]. In some countries, *P. aeruginosa* possessing MBLs constitute nearly 20% of all nosocomial isolates, whereas in other countries the number is still comparatively small. In recent years MBL genes have spread from *P. aeruginosa* to *Pseudomonas* spp., *Acinetobacter* spp., and Enterobacteriaceae, and a clinical scenario appears to be developing that could simulate the global spread of extended-spectrum beta-lactamases. Moreover, given that MBLs will hydrolyse virtually all classes of beta-lactams and that we

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are several years away from the implementation of a ‘therapeutic inhibitor’, their continued spread could be a clinical catastrophe.

74.2 Summary of Methods

This method focuses on the ability to multiplex in one reaction tube for detection of all three types of MBL genes; *bla*$_{\text{imp4}}$, *bla*$_{\text{spm}}$ *and bla*$_{\text{vim}}$.

The assay utilises 5′ nuclease-based real-time PCR comprising two primers and one probe for each MBL gene target, each labelled with a different fluorophore. *bla*$_{\text{imp4}}$ has been chosen as this appears to be the predominate MBL present in the Australian bacterial population [2].

74.3 Acceptable Specimens

Pure bacterial cultures are the preferred samples for this PCR.

74.4 Sample Extraction

Plugged tips must be used for all pipetting steps. Change powder-free gloves regularly. DNA extraction is performed in the Class II Biological Safety Cabinet. Only trained staff with demonstrated competence may perform the test.

Extract DNA following the Instagene matrix (BioRad Laboratories) procedure for bacterial isolates. Briefly, to 200 μl of Instagene matrix (mix well prior to addition) is added a small loop of colony of the organism of interest. Incubate at 56°C for 15–30 min with shaking in the Eppendorf thermomixer. Place the tube in a 99°C heat block with shaking (second thermomixer) for 8 min. Spin at 13,000 rpm for 2–3 min (this pellets the Chelex resin and cellular material). Use the supernatant in the PCR reaction.

For each assay run, one positive *bla*$_{\text{imp}}$, *bla*$_{\text{spm}}$ *and bla*$_{\text{vim}}$ control and one reagent negative control and no DNA control (5 μl of sterile distilled autoclaved water) would be typical. After extraction each nucleic acid suspension is held at 4°C for up to 4 h or frozen at –20°C or lower until required. They may be thawed for use as required but repeat the last centrifugation step if reusing the Instagene DNA preparation.

74.5 MBL Multiplex PCR Master Mix

Master Mix is prepared fresh and as a bulk mix. Volumes will vary depending on the numbers of samples and controls to be tested. The master mix is added to capillaries just prior to adding DNA extract.

74.6 Reactants for 1x Mix

Fast Start DNA Master mix *PLUS* Hybridisation probes reaction mix (Roche) (10x) (10 μl 1a into 1b. Store at 4°C for 1 week)		5.0 μl
Bla_{imp4}-F	*(20 μM)*	0.625 μl
Bla_{imp4}-R	*(20 μM)*	0.625 μl
Bla_{imp4} probe (FAM) *(20 μM)*		0.25 μl
Bla_{spm} -F	*(20 μM)*	0.625 μl
Bla_{spm} -R	*(20 μM)*	0.625 μl
Bla_{spm} probe (TxR) *(20 μM)*		0.25 μl
Bla_{vim}- F	*(20 μM)*	0.625 μl
Bla_{vim} -R	*(20 μM)*	0.625 μl
Bla_{vim} probe (TET) *(20 μM)*		0.25 μl
Uracil-n-glycosylase (UNG) (LC Roche Cat. No. 03539806) (optional at 1 μl /reaction)		
Water nuclease free		*10.5 μl*
Total per reaction tube		20.0 μl
Add DNA extract		5.0 μl
Total per tube (μl)		**25.0** μl

The primers and probe are held at -30°C in small volume aliquots (5 μl probe and 10 μl each primer) to exclude repeated freeze/thaw cycles and potential probe hydrolysis. This bulk mix is made up in a sterile or nuclease free 1.5 ml eppendorf tube using a separate plugged tip for each solution. Briefly hand mix the master mix and spin for 5 s to assemble all components into the bottom of the tube. Aliquot the master mix to the capillaries and add specimen or control sample extract. Keep cool.

74.7 Primers and Probe Sequences

Bla_{imp4} – F	5′ GGCAGTATTTCCTCTCATTT 3′
Bla_{imp4}– R	5′ GCAGCTCATTAGTTAATTCAG 3′
Bla_{spm} –F	5′ GGGTACGCAAACGCTTATGG 3′
Bla_{spm} – R	5′ CCGTGCCGTCCAAATGAAAG 3′
Bla_{vim} – F	5′ CGCGGAGATTGAGAAGCAAA 3′
Bla_{vim} – R	5′ AGCCGCCCGAAGGACATC 3′

These are purchased from Proligo Australia (Lismore) at a concentration of 100 μM. Aliquot small volumes of 20 μM solutions (1:5 dilution in nuclease free water) and store labelled –20°C.

Bla_{imp4} 5′ (6- FAM) CATAGTGACAGCACGGGCGGAAT (BHQ1) 3′
Bla_{spm} 5′ (Texas Red) TTGGGTGGCTAAGACTATGAAGCCGA (BHQ2) 3′
Bla_{vim} 5′ (TET) TTGGACTTCCTGTAACGCGTGCA (BHQ1) 3′

These are purchased from Biosearch Technologies, Novato CA. USA. and are diluted to a concentration of 100 μM. Aliquot small volumes of 20 μM solutions as a working solution (1:5 dilution in nuclease free water) and store labelled at –20°C.

74.8 Addition to Reaction Tubes

Add 20 μl of this bulk master mix to the lid section of the SmartCycler reaction tubes. Load 5 μl of specimen/control sample extract to the lid section and immediately close the lid. Spin the reaction tubes for 10 s at a speed of 3000 rpm in the dedicated microfuge to ensure all solution is within the reaction tube. Let stand at room temperature for 10 min to allow UNG (if incorporated) to destroy any previously amplified product (potential source of contamination).

74.9 PCR Amplification and Product Detection

This is performed on a Cepheid SmartCycler II Instrument, using SmartCycler software and dye set FTTC25 (FAM, TET, TXR, Cy5).

The reaction tubes are initially spun and then placed into the appropriate ‘site’ in the SmartCycler. You can use any of the sites as long as you set up the software to match. Ensure tubes are in the correct order:

The programme cycling profile is Activation 1x at 95°C × 10 min, Amplification is 30 cycles at 95°C × 10 s, 60°C × 30 s single acquisition, cool at 40°C × 30 s. A low cycle number of cycles is fine when using a culture isolate. Total cycling time is around 40 min.

74.10 Interpretation and Reporting of PCR Results

Print each of the FAM, TET, TxR dyes and analyse and report results. If any of the controls do not meet the expected results the run is considered invalid and samples must be retested and/or phenotypic analyses performed.

74.11 Quality Control and Validation Data

Extracted nucleic acid from previously PCR-positive bacterial isolates may be used as a positive control. Any positive control should be diluted to a concentration providing a cycle-threshold (Ct) value of approximately 25 cycles in the real-time PCR.

200 μl of Instagene matrix is subject to the extraction procedure and is used as a negative control.

This assay has been validated against the CDS method of antibiotic susceptibility testing [5]. It was presented at the annual ASM Conference in Melbourne 2008 as a poster.

To date all isolates tested have matched both phenotypic and multiplex PCR results 100% and bacterial isolates tested containing an MBL include *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Morganella* sp. isolate. The majority have contained an *imp* gene but only one has contained a *vim* gene. Allelic variants for the MBL types $bla_{\text{SPM-1}}$, $bla_{\text{GIM-1}}$, and $bla_{\text{SIM-1}}$ have not been detected yet. A SYBR green based multiplex PCR utilising melt-curve analysis has been published [1] and may prove useful for identification of MBL genes contained within resistant organisms.

References

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