isolates that are identified as *A. baumannii* by commercial identification methods, but that fail to yield a PCR product, may be identified tentatively as genomic sp. 3 if they grow at 41°C but fail to grow at 44°C.

The *gyrB* PCR method is robust and reproducible, and can yield a result in <2.5 h. Its simplicity means that it can be employed readily in most laboratories, where it should contribute to a better understanding of the epidemiology and clinical significance of the two most important *Acinetobacter* species.

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RESEARCH NOTE

Specific detection of bla_{VIM} and bla_{IMP} metallo-β-lactamase genes in a single real-time PCR

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ABSTRACT

This study describes the development of a real-time PCR protocol for rapid detection of the most common bla_{VIM} (bla_{VIM-1}, bla_{VIM-2}, bla_{VIM-3}, bla_{VIM-4}, bla_{VIM-5}, bla_{VIM-6}, bla_{VIM-10}, bla_{VIM-11}, bla_{VIM-12}) and bla_{IMP} (bla_{IMP-1}, bla_{IMP-2}, bla_{IMP-6}, bla_{IMP-8}, bla_{IMP-10}, bla_{IMP-15}, bla_{IMP-19}, bla_{IMP-20}) genes in a single reaction. The genes were specifically detected and clearly differentiated into four groups, i.e., (i) bla_{VIM-1}-like (bla_{VIM-1}, bla_{VIM-4}, bla_{VIM-5}, bla_{VIM-12}); (ii) bla_{VIM-2}-like (bla_{VIM-2}, bla_{VIM-3}, bla_{VIM-6}, bla_{VIM-10}, bla_{VIM-11}); (iii) bla_{IMP-1}-like (bla_{IMP-1}, bla_{IMP-6}, bla_{IMP-10}); and (iv) bla_{IMP-2}-like (bla_{IMP-2}, bla_{IMP-8}, bla_{IMP-15}, bla_{IMP-19}, bla_{IMP-20}), by melting curve analysis of the real-time PCR products. The protocol was used to screen positive bla_{VIM-1}, bla_{VIM-2} and bla_{IMP-1} control strains, 70 Gram-negative isolates resistant to carbapenems, and 30 Gram-negative isolates susceptible to carbapenems (negative controls).

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The VIM and IMP classes of metallo-β-lactamases include at least 14 and 23 different enzymes, respectively (http://www.lahey.org/studies/other/htm), and these enzymes are increasingly being detected in clinical isolates worldwide [1]. This study describes a real-time PCR assay for the specific detection of bla_{VIM} and bla_{IMP} genes in Gram-negative bacteria that allows amplification, detection and product identification in <1 h.

Sixty *Pseudomonas aeruginosa* isolates, 36 *Klebsiella pneumoniae* isolates and four *Pseudomonas putida* isolates recovered from various clinical specimens were used to evaluate the real-time PCR protocol (see below). The Vitek2 system (bioMérieux, Hazelwood, MO, USA) and Etests (AB Biodisk, Solna, Sweden) were used for bacterial identification and detection of resistance to imipenem and meropenem [2]. The presence of a metallo-β-lactamase was tested using MBL Etests [3]. DNA was extracted using a high-pure PCR Template Preparation Kit (Roche, Mannheim, Germany) from single bacterial colonies growing on MacConkey agar. The kit includes a buffer for the removal of potential inhibitors. The quality and the quantity of extracted DNA were determined using a Genova MK3 analyser (Jenway Ltd, Dunmow, UK).

The real-time PCR assay utilised the LightCycler 2.0 platform (Roche). Primers and HybProbes were designed and synthesised by TIB MolBiol Syntheselabor GmbH (Berlin, Germany), based on published sequences corresponding to the most common bla_{VIM} and bla_{IMP} gene families (Table S1, see Supplementary material). HybProbes were used for the specific detection and identification of the bla_{VIM} and bla_{IMP} genes. Two sequence-specific oligonucleotide probes labelled with different dyes, termed the anchor and sensor probes, hybridise to target sequences on the amplified DNA, bringing the two dyes into close proximity. The anchor dye (fluorescein) is excited by a light-emitting diode, and the energy emitted then excites the sensor dye attached to the second probe, which emits fluorescent light at a different wavelength. The latter signal is measured by the LightCycler detection system.

Sequence alignment of the most common bla_{VIM} genes (bla_{VIM-1}, bla_{VIM-2}, bla_{VIM-3}, bla_{VIM-4}, bla_{VIM-5}, bla_{VIM-6}, bla_{VIM-10}, bla_{VIM-11}, bla_{VIM-12}) revealed that these could be divided into two groups, named bla_{VIM-1}-like (bla_{VIM-1}, bla_{VIM-4}, bla_{VIM-5}, bla_{VIM-12}) and bla_{VIM-2}-like (bla_{VIM-2}, bla_{VIM-3}, bla_{VIM-6}, bla_{VIM-10}, bla_{VIM-11}). The two groups can be distinguished by amplification using the same forward primer (VIM-fw 5′-GTACGCATCACCGTCGACAC), but different reverse primers. The bla_{VIM-1}-like genes are only amplified using reverse primer VIM-spec1-re (5′-TGACGGGACGTATACAACCAGA), whereas the bla_{VIM-2}-like genes are only amplified using reverse primer VIMspec2-re (5′-AGACGGGACGTACACAACTAAG). In both cases, the amplification product is 172 bp in size, but the products can be distinguished using HybProbes and melting curve analysis following the PCR. While the VIM-Anch (5′-GGTGCTGCGCATTCGACCGACA-FL) Hybprobe perfectly matches all these genes, the VIM-Sen (5′-LCRed640-TTCGGTCCAGTAGAACTCTTCTATCC-PH) Hybprobe perfectly matches the bla_{VIM-2} group, but has a mismatch with the bla_{VIM-1} group, resulting in different melting profiles.

In order to amplify the most common bla_{IMP} genes (bla_{IMP-1}, bla_{IMP-2}, bla_{IMP-6}, bla_{IMP-8}, bla_{IMP-10}, bla_{IMP-15}, bla_{IMP-19}, bla_{IMP-20}), forward primer IMP-fw (5′-AAGTTAGTCA(A/C)TTGGTTTGTGGAGC) and reverse primer IMP-as (5′-CAAACCACTACGTTATCT(G/T)GAGTGTG) were used to produce a 269-bp PCR product. The bla_{IMP-13} gene is not amplified by these primers. The two HybProbes (IMP-Anch, 5′-AGTTCATTTGTTAATTCAGATGCATACGTGGG-FL and IMP-Sen, 5′-LCRed640-ATAGATCGAGAATTAAGCCACTCTATTCC-PH) were designed to group the bla_{IMP} genes into two categories, i.e., bla_{IMP-1}-like genes (bla_{IMP-1}, bla_{IMP-6}, bla_{IMP-10}) and bla_{IMP-2}-like genes (bla_{IMP-2}, bla_{IMP-8}, bla_{IMP-15}, bla_{IMP-19}, bla_{IMP-20}). While the IMP-Anchor probe perfectly matches both groups, the IMP-sensor probe perfectly matches with the bla_{IMP-1} group, but has a mismatch with the bla_{IMP-2} group, resulting in different melting profiles.

Real-time PCR was performed in glass capillaries with a final volume of 20 μL. Each PCR assay contained 0.5 μM each primer, 0.2 μM each probe, 1 U of uracil-DNA-glycosylase, 2 μL of LightCycler FastStart DNA Master Mix (Roche),

Table 1. Summary of antimicrobial susceptibility tests, Etests for metallo-β-lactamase production, and real-time PCR assays

Isolates	No. of isolates	Carbapenem resistance	Etest for metallo-β-lactamases	Real-time PCR
Pseudomonas aeruginosa	35	Resistant	Positive	bla_{VIM-2} like
P. aeruginosa	10	Resistant	Negative	Negative
P. aeruginosa	15	Sensitive	Negative	Negative
Pseudomonas putida	1	Resistant	Positive	bla_{VIM-2} like
P. putida	3	Sensitive	Negative	Negative
Klebsiella pneumoniae	21	Resistant	Positive	bla_{VIM-1} like
K. pneumoniae	3	Resistant	ND	bla_{VIM-1} like
K. pneumoniae	12	Sensitive	Negative	Negative

3 mM $MgCl_2$ and 1 μL of DNA extract. Thermocycling and detection were performed in the LightCycler 2.0 instrument, with 35 cycles of 95°C for 10 s, 48°C for 7 s and 72°C for 15 s. Fluorescence corresponding to specific amplified targets was measured at 640 nm after each cycle. In order to identify the PCR products, melting curve analysis was performed by heating to 95°C for 3 s, annealing at 48°C for 45 s, and then slowly heating from 48°C to 95°C with a step-mode acquisition of fluorescence at 640 nm.

Control strains comprised a *K. pneumoniae* strain with a bla_{VIM-1} gene and two *P. aeruginosa* strains with a bla_{VIM-2} and a bla_{IMP-1} gene, respectively. A single PCR containing primers and probes for the bla_{IMP} and bla_{VIM} genes was performed for each test organism. The melting curve analysis for the bla_{VIM-1} and bla_{VIM-2} control strains generated T_m values of 60.28°C (average of 25 separate runs; standard deviation 0.51°C) and 65.12°C (average of 25 separate runs, standard deviation 0.75°C), respectively. The lower melting peak for the bla_{VIM-1} control strain is explained by a mismatch of the VIM sensor probe with the DNA target. The melting curve analysis of the bla_{IMP-1} control strain generated a T_m value of 52.24°C (average of 20 separate runs, standard deviation 0.37°C). Amplification of a bla_{IMP-2}-like gene would have generated a T_m value of <52°C because of the mismatch with the IMP sensor probe.

When the assay was used to screen 100 isolates of Gram-negative bacteria recovered from clinical samples, all *P. aeruginosa* and *P. putida* isolates resistant to imipenem/meropenem that produced a metallo-β-lactamase were found to carry a bla_{VIM-2}-like gene, while a bla_{VIM-1}-like gene was detected in all carbapenem-resistant isolates of *K. pneumoniae* (Table 1). The real-time PCR results were confirmed by electrophoresis of the products. No PCR products were amplified from the carbapenem-susceptible isolates.

Extensive spread of bla_{VIM} and bla_{IMP} genes among clinically important Gram-negative bacteria is now being observed worldwide, with most of these genes being located on integrons in combination with other resistance genes [1,4,5]. The real-time PCR protocol described above can be used to screen for the presence of most common bla_{VIM} and bla_{IMP} genes so that their epidemiological spread can be monitored.

SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online at http://www.blackwell-synergy.com:

Table S1. Results of sequence alignments for the real-time PCR primers and the most commonly described bla_{VIM} and bla_{IMP} genes

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