RESEARCH LETTER

Development and evaluation of polymerase chain reaction assay to detect *Burkholderia* genus and to differentiate the species in clinical specimens

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Abstract

Molecular-based techniques are becoming desirable as tools for identification of infectious diseases. Amongst the *Burkholderia* spp., there is a need to differentiate *Burkholderia pseudomallei* from *Burkholderia cepacia*, as misidentification could lead to false treatment of patients. In this study, conventional PCR assay targeting three genes was developed. Primers were designed for the amplification of *Burkholderia* genus-specific *groEL* gene, *B. pseudomallei*-specific *mprA* gene and *B. cepacia*-specific *zmpA* gene. The specificity and sensitivity of the assay was tested with 15 negative control strains and 71 *Burkholderia* spp. isolates including positive controls *B. pseudomallei* K96243 and ATCC *B. cepacia* strain. All *B. pseudomallei* strains were positive for *groEL* (139 bp) and *mprA* (162 bp), indicating a sensitivity of 100%. All *B. cepacia* strains produced amplicons for detection of *groEL* and *zmpA* (147 bp). Specificity using negative strains was 100%. In this study, a PCR assay specific for the detection of *Burkholderia* spp. and differentiation of the genus *B. pseudomallei* and *B. cepacia* was developed. The conventional assay has to be performed separately for each species due to the similar size of the PCR products amplified. This format may therefore be recommended for use as a diagnostic tool in laboratories where real-time PCR machines are not available. However, the real-time PCR was able to detect and differentiate the genus and species in single duplex assay.

Introduction

The genus *Burkholderia* consists of more than 30 species of Gram-negative bacilli, nonspore forming and oxidase-positive soil saprophytes. *Burkholderia pseudomallei* and *Burkholderia cepacia* complex are known human pathogens. *Burkholderia mallei* causes glanders in horses and *Burkholderia thailandensis* is a nonpathogenic bacterium. All *Burkholderia* spp. are motile except for *B. mallei* (Bossi *et al*., 2004). *Burkholderia pseudomallei* causes melioidosis in humans, which resembles glanders and is predominant in South-East Asia and Northern Australia. *Burkholderia cepacia* has been recognized as a major opportunistic pathogen in cystic fibrosis, necrotizing pneumonia and chronic granulomatous diseases in humans (Isles *et al*., 1984). In addition, *B. cepacia* causes urinary tract infections, wound infections and endocarditis (Speller *et al*., 1971).

To date, various diagnostic methods such as culture (Anuntagool *et al*., 1993), serology (Illeri, 1965; Walsh *et al*., 1994; Chentamarakshan *et al*., 2001) and molecular detection methods (Rattanatongkom *et al*., 1997; Sura *et al*., 1997; Woo *et al*., 2002) have been developed for identification of *Burkholderia* spp. either from environmental or clinical samples. Although culture is known as the 'gold standard' for the detection of *Burkholderia* spp., it is time-consuming, often taking up to 48 h. Early confirmative detection of *B. pseudomallei* is essential for septicemic cases in which fatality can occur within 24–48 h. Pathogen detection via molecular methods such as PCR is becoming useful in the rapid identification of clinically important pathogens due to its ability to determine the definitive causative agent within few hours. Bauernfeind *et al*. (1998) developed a PCR assay to differentiate *B. pseudomallei* from *B. mallei* using the primers designed for 23S rRNA gene. Among the genes commonly targeted for the detection of *Burkholderia* spp. in a singleplex, multiplex or real-time PCR

have been 16S rRNA gene, ribosomal protein subunit S21 (*rpsU*) and flagellin C (*fliC*) (Hagen *et al.*, 2002; Tomaso *et al.*, 2005), type three secretion system (TTS1) (Rattanatongkom *et al.*, 1997) and recombinant A (*recA*) (Mahenthiralingam *et al.*, 2000; Payne *et al.*, 2005).

In this study, a PCR assay specific for the detection of *Burkholderia* spp. and differentiation of the genus *B. pseudomallei* and *B. cepacia* was developed. The assay is in the conventional format, which has to be performed separately for each species due to the similar size of the PCR products amplified. This format may therefore be recommended for use as a diagnostic tool in laboratories where real-time PCR machines are not available. However, this assay was able to detect and differentiate the genus and species in a single duplex assay using real-time PCR. These PCR assays were developed targeting three different genes: *groEL* gene for the general detection of *Burkholderia* genus, *mprA* gene of *B. pseudomallei* and *zmpA* gene of *B. cepacia*. Direct detection in clinical specimens from suspected melioidosis patients was also performed and evaluated with culture and biochemical characterization.

Materials and methods

Bacterial strains

Bacterial strains used in this study were obtained from the Medical Microbiology Diagnostic Laboratory, University Malaya Medical Centre (UMMC, Kuala Lumpur) and Hospital Tengku Ampuan Afzan (HTAA, Kuantan, Pahang) and included 65 strains of *B. pseudomallei*, three isolates of *B. cepacia*, one *B. thailandensis* strain and 15 negative control strains of *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., *Acinetobacter* spp., *Pseudomonas stutzeri*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae* and *Mycobacterium tuberculosis*. In addition, *B. pseudomallei* K96243 and *B. cepacia* ATCC 25416 were used as reference strains. All *Burkholderia* and negative control strains were isolated from clinical sources and culture collections were confirmed using biochemical characterization and API 20E assay (Bio-Merieux, France, UMMC).

Clinical samples

Blood samples from patients suspected of having melioidosis were obtained from in patients with septicemia at UMMC. All blood samples were subjected to direct PCR for amplification of *B. pseudomallei* genes specifically and also for culture and biochemical characterization. Serum samples collected retrospectively from patients confirmed for melioidosis were also included in the PCR amplification. These samples were collected over a period of 2 months and were confirmed as melioidosis-positive by immunofluorescent antibody assay (Vadivelu & Puthucheary, 2000). All clinical specimens were stored at $-70\,^{\circ}$C for the duration of the study.

DNA preparation

DNA from culture samples was prepared by a simple boiling method (Merritt *et al.*, 2006). Culture samples obtained from the diagnostic laboratory were subcultured on nutrient agar and incubated at 37 °C overnight. DNA extraction from culture samples was done as described with some modifications. A single colony from the overnight culture was picked using a flamed wire loop and suspended in 100 μL of sterile distilled water. The bacterial suspension was then boiled at 100 °C for 10 min followed by centrifugation at 13 000 ***g*** for 1 min and the supernatant containing the DNA was aliquoted and stored at $-20\,^{\circ}$C for the course of the study. Extraction of DNA from blood samples was performed according to the protocol provided with the Qiagen Blood Mini Amp Kit (Qiagen).

Primer design using bioinformatics tools

Three sets of primers were designed, each one targeting *groEL* (chaperonin) (gro1 and gro2) of *Burkholderia* genus, *mprA* (serine metalloprotease) (mpr1 and mpr2) gene of *B. pseudomallei* and *zmpA* (zinc metalloprotease) (zmp1 and zmp2) gene of *B. cepacia*, respectively (Table 1, Patent Ref: *PI 20083144*). All gene sequences were obtained from the

Table 1. Details of primers used in this study

Name	Orientation	Sequence	Length	Position	Product size (bp)	T_m (°C)	GC (%)
gro1	5′–3′	CTG GAA GAC ATC GCG ATC	18	865–882*	139	56.0	55.6
gro2	5′–3′	CGT CGA TGA TCG TCG TGT T	19	985–1003*		58.0	52.6
mpr1	5′–3′	TCT CCG ATA GCC GCC TTG	18	34–51†	162	58.0	61.1
mpr2	5′–3′	CGT ATC ACA TCG CAT CGC	18	178–195†		56.0	55.6
zmp1	5′–3′	ACC CTC GCG AGT CTC AG	17	40–56‡	147	56.0	64.7
zmp2	5′–3′	TTG TGG CCC GGC GAA CTT	18	169–186‡		58.0	61.1

*Nucleotide sequence based on gene accession number AF287633.
†Nucleotide sequence based on gene accession number AF254803.
‡Nucleotide sequence based on gene accession number AY143552.
All primers were patented, Patent Ref: *PI 20083144*.

National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov), and analyzed using the BLAST and CLUSTALW programs to reveal the conserved as well as unique regions of the targeted genes. The GenBank accession numbers for *groEL*, *mprA* and *zmpA* were AF287633, AF254803 and AY143552, respectively. The primers were designed with similar melting temperatures to enable conversion of standard PCR to multiplex PCR in future. Each of the sequences was then analyzed using BLAST to ascertain the specificity of the primers for the possibility of cross-reaction with other closely related organisms. The primer sequences were also analyzed for the presence of secondary structures using the OLIGO ANALYZER software. Primers that satisfactorily fulfilled the basic criteria were chosen and synthesized by Helix Biotech (Sigma Proligo, France).

Optimization of PCR

All PCR reactions were set up in 0.5-μL flat cap Eppendorf microcentrifuge tubes. Optimization parameters included $MgCl_2$ concentration, annealing temperature and the number of PCR cycles. $MgCl_2$ concentrations were optimized using 1.0 mM, 1.5 mM and 2.5 mM and the annealing temperature was set at 52 °C (predicted, based on melting temperature of primers) and number of cycles randomly at 35. The annealing temperature was then optimized using gradient PCR at temperatures ranging from 50 to 60 °C. Finally, PCR cycles were optimized using 25, 30 and 35 cycles. The rest of the parameters were followed within the range recommended by standard PCR protocol: 1 × buffer, 0.2 μM of each of the primers, 200 μM of dNTP, 1.25 U of Taq DNA Polymerase recombinant and 5 ng μL^{-1} of DNA for 50 μL of final reaction volume. PCR reactions were performed using a BioRad DNA thermal cycler.

Conventional PCR assay

The primers were initially tested using the targeted control organisms and closely related organisms. The PCR reactions were carried out in a final volume of 50 μL containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, 1.25 U of Taq DNA Polymerase and 5 μL of DNA template and distilled water. Initial denaturation was performed at 94 °C for 5 min, followed by amplification comprising 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 45 s. A further 2-min final extension at 72 °C was carried out following the final cycle. The amplified PCR products were analyzed using 1.5% agarose gel (Promega) electrophoresis in 1 × TBE buffer at 90 V for 1 h and visualized using ethidium bromide staining under UV illumination. The positive PCR products were purified using Wizard PCR Purification Kit (Promega) and confirmed by sequencing (Research Biolabs Sdn. Bhd, Singapore). The limit of dilution was determined by subjecting the DNA of the targeted organisms to PCR after 10-fold serial dilutions to produce a DNA concentration ranging from 10 μg mL^{-1} to 10 fg mL^{-1}.

Real-time PCR assay

Real-time duplex PCR amplification and melt curve analysis were carried out in an iQ5 real-time PCR detection system (BioRad Laboratories, Hercules, CA). QuantiTect SYBR green PCR kit (Qiagen) was used for amplification with 0.3 μM of *mprA* and 0.2 μM of *zmpA* primers. The PCR was performed with the following cycling protocol. Initial denaturation for 15 min at 95 °C was followed by 30 cycles with 15 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C. Fluorescence data were captured at the elongation step of each cycle. Following amplification, melt curves were acquired by increasing the temperature from 65 to 95 °C at the rate of 0.5 °C $10\,s^{-1}$, with continuous measurement of the fluorescence.

Results

In general, all three query gene sequences retrieved from the GenBank and analyzed by BLAST were correct with an exact match of 100% identity. CLUSTALW alignment revealed that the *groEL* gene sequence of *B. pseudomallei* was highly homologous to *B. mallei*, *B. thailandensis* and *B. cepacia*, with a score of 99%, 97% and 95%, respectively. The alignment scores of other organisms such as the *Pseudomonads*, *Xanthomonas campestris*, *Bordetella pertussis* and *Ralstonia picketti* displayed a distant relation to *Burkholderia* spp. Therefore, the regions of *groEL* appropriate for primer design were targeted at the part where there was 100% identity of bases among *Burkholderia* spp. and vast variation with other organisms. The *mprA* gene sequenced was not aligned with any other organisms as no database was found for a similar gene in other organisms. The *zmpA* of *B. cepacia* was aligned with that of *B. pseudomallei*. Alignment results revealed an identity of 86% between these two sequences. Thus, the regions that displayed significant nucleotide variation within *zmpA* sequences of these two organisms were chosen for primer design.

Using conventional PCR assay, the gro1 and gro2 primers amplified a 139-bp product from all *Burkholderia* spp., whereas the 162- and 147-bp mpr and zmp products were amplified from *B. pseudomallei* and *B. cepacia*, respectively (Fig. 1). All 66 *B. pseudomallei*, one *B. thailandensis* and four *B. cepacia* clinical isolates were positive for the *groEL* gene, indicating successful detection of the genus *Burkholderia*. All 65 *B. pseudomallei* isolates and K96243 strain were positive for the detection of *mprA* gene. Similarly, all three *B. cepacia* isolates and ATCC 25416 strain were positive for *zmpA* gene. Sequence analysis of the PCR products from the amplification of *groEL*, *mprA* and *zmpA* matched the published gene sequences in the NCBI website.

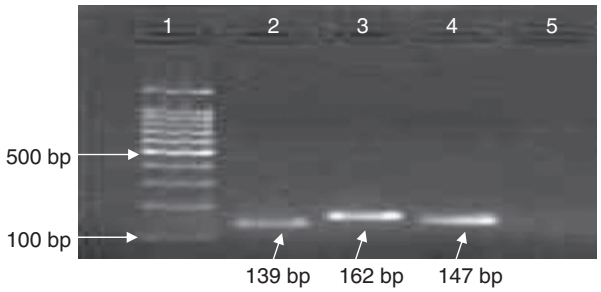

Fig. 1. Amplification of *groEL* (139 bp), *mprA* (162 bp) and *zmpA* (147 bp). Lane 1, 100 bp ladder (Favorgen); lane 2, amplification of *groEL* in *Burkholderia pseudomallei* K96243; lane 3, amplification of *mprA* in *B. pseudomallei* K96243; lane 4, amplification of *zmpA* in ATCC *Burkholderia cepacia*; lane 5, negative control (without template DNA).

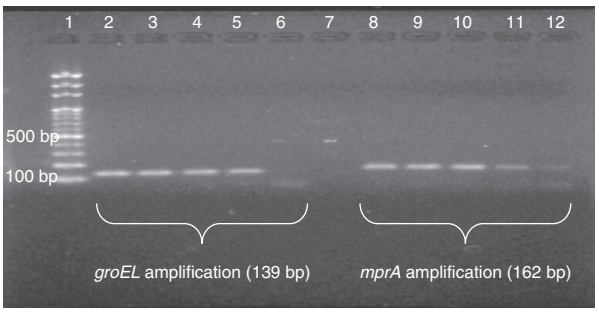

Fig. 2. Limit of detection for *groEL* (139 bp) and *mprA* (162 bp) targeted PCR assay. Lane 1, 100 bp ladder (source); lanes 2 and 8, neat *Burkholderia pseudomallei* DNA (25 μg mL^{-1}); lanes 3 and 9, dilution 10 μg mL^{-1}; lanes 4 and 10, dilution 10 ng mL^{-1}; lanes 5 and 11, dilution 10 pg mL^{-1}; lanes 6 and 12, dilution 10 fg mL^{-1}; lane 7, negative control (without DNA template).

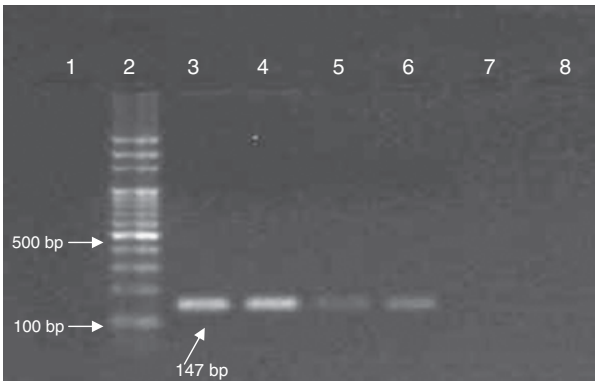

Fig. 3. Limit of detection for *zmpA* (147 bp) targeted PCR assay. Lane 1, empty; lane 2, 100-bp ladder; lane 3, neat *Burkholderia cepacia* DNA (25 μg mL^{-1}); lane 4, dilution 10 μg mL^{-1}; lane 5, dilution 10 ng mL^{-1}; lane 6, dilution 10 pg mL^{-1}; lane 7, dilution 10 fg mL^{-1}; lane 8, negative control (without DNA template).

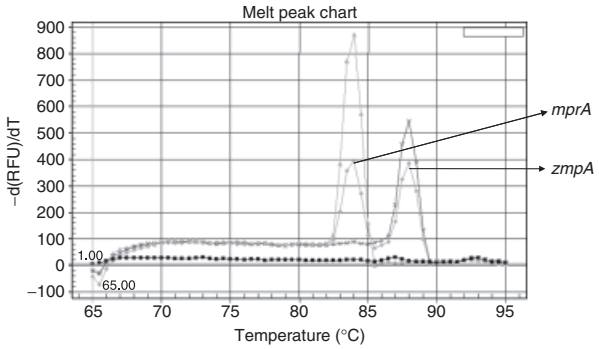

Fig. 4. Melting curves for the *mprA* and *zmpA* genes based on the SYBR green PCR. The mean of the melting peak for the 162-bp amplicon of *mprA* = T_m 84 °C and for 147-bp amplicon of *zmpA* = T_m 88 °C. No primer dimers were observed in the amplified product.

The negative control strains did not yield any PCR product, suggesting that the primers were highly specific for the different *Burkholderia* spp. In addition, no cross-reactions were observed within the *Burkholderia* spp. The *mprA* and *zmpA* genes were correctly amplified in the targeted strains, indicating a specificity of 100%. The limit of detection assay demonstrated that the *groEL* and *zmpA* PCR assay was sensitive at 10 pg mL^{-1} DNA, whereas *mprA* PCR assay was sensitive at 10 fg mL^{-1} (Figs 2 and 3).

The PCR assay using DNA obtained from blood samples revealed successful amplification of *B. pseudomallei* in two of the 18 samples tested. On comparison with culture and API 20 NE results, these two PCR-positive samples were also positive for *B. pseudomallei* by culture and API 20 NE. The PCR-negative samples were also negative on culture, indicating sensitivity and specificity of 100%. However, none of the serum samples produced positive amplicons for any of the three primer sets.

Duplex real-time PCR using SYBR green was performed using *mprA* (162 bp) and *zmpA* based on the melting curve analysis of amplified products. These primers allowed the amplification of PCR products with distinct melting temperature values, resulting in the formation of two distinct peaks representing the two targets. The 167-bp amplicon of *mprA* (T_m 84 °C) could be clearly separated from the 147-bp amplicon of *zmpA* (T_m 88 °C) (Figs 4 and 5). No primer dimers were observed in the amplified product, which indicates the specificity of the primers.

Discussion

In this study, a conventional PCR assay was developed for the detection of *Burkholderia* genus and also for differentiation of the two clinically important human pathogens, *B. pseudomallei* and *B. cepacia*. Using bioinformatics tools, this assay incorporated detection of *groEL* gene, specific for the genus *Burkholderia*, *mprA* gene, specific for *B. pseudomallei*, and *zmpA* genes specific for *B. cepacia*. The *groEL* gene encodes an immunogenic protein of *Burkholderia* that

assists in a proper protein-folding mechanism (Woo *et al.*, 2001). BLAST analysis revealed that *groEL* is present in *B. mallei*, *B. pseudomallei*, *B. cepacia*, *Burkholderia vietnamiensis* and *B. thailandensis* among the *Burkholderiaceae*. Moreover, this gene sequence is highly conserved among all *Burkholderia* spp. and therefore shares high homology of amino acid identity, as reported by Woo *et al.* (2001). The *mprA* gene encodes for a specific novel metalloprotease for *B. pseudomallei* that has proteolytic and cytotoxic activity (Lee & Liu, 2000). In this study, there was a 100% sensitivity and specificity for detection of this gene. This is in agreement with a study conducted by Neubauer *et al.* (2007). The *mprA* gene was targeted for detection of *B. pseudomallei* from naturally infected dromedary and showed a sensitivity and specificity of 100%. The *zmpA* gene that encodes for zinc metalloprotease was known originally as *Pseudomonas cepacia* protease. It has the capability of cleaving biologically important substances such as gelatin, hide powder and human collagen types I, IV and V (McKevitt *et al.*, 1989).

In this study, the PCR assay was also performed on DNA obtained directly from clinical specimens such as blood and body fluids. The positive control included in this assay was DNA extracted from *B. pseudomallei* control strain. It is not possible to include a positive blood sample in every PCR assay. Furthermore, the two of the 18 blood specimens that were positive by PCR were also found to be positive by conventional culture and biochemistry. The PCR-negative blood samples also produced consistent negative results by culture and biochemistry. This suggests that there was no circulating *B. pseudomallei* in the blood samples that were PCR-negative, and the probability of the presence of inhibitory substances in the blood and other body fluids can be ruled out as results were confirmed using the 'gold standard' culture. However, we treat this data with caution as the number of samples studied was small. A larger sample size would have been more desirable.

Although many studies have attempted to identify *Burkholderia* spp. by means of PCR, none of these was developed for the detection of *Burkholderia* genus in conjunction with differentiation of *B. pseudomallei* and *B. cepacia*, as done in our study. The use of *mprA* and *zmpA* genes specifically to identify *B. pseudomallei* and *B. cepacia*, respectively, thus differentiating these two species, has not been reported elsewhere. Other studies have only attempted to differentiate *B. mallei* from *B. pseudomallei*. These include development of PCR for differentiation of *B. mallei* from *B. pseudomallei* targeting *bimA* (Ulrich *et al.*, 2006) and 16S rRNA gene (Gee *et al.*, 2003) and differentiation of the genomovars in *B. cepacia* complex individually, using the *recA* gene (Payne *et al.*, 2005). However, even these assays were unable to distinguish the *Burkholderia* spp. due to presence of conserved regions. An *mprA*-based PCR assay for specific detection of *B. pseudomallei* was reported recently by Neubauer *et al.* (2007). However, this assay differed from ours as the detection of *B. pseudomallei* in their study was intended for animal samples involving different primers. When comparing the limit of detection of the PCR assay, our assay displayed a detection limit of 10 pg mL^{-1}, in contrast to previous studies that had limit of detection at only 10 ng mL^{-1} (Bauernfeind *et al.*, 1998; Lee *et al.*, 2005; Ulrich *et al.*, 2006).

Amplification in serum samples revealed negative results. This suggests that although the serum samples were obtained from melioidosis-positive patients, the prevalence of circulating bacteria in serum was low as compared with whole blood. Another likely explanation could be that the serum obtained from patients was from a later date of infection, indicated by the presence of antibody, therefore resulting in the clearance of the bacteria. Additional possibilities for negative amplification include incorrect PCR mixture, degradation of DNA due to long-term storage, poor DNA polymerase activity or presence of inhibitory substances in the sample. The detection of *B. pseudomallei* from clinical specimens such as blood and serum could be improved using real-time PCR assay or internal control.

In the current study, the primers selected for *mprA* (162 bp) and *zmpA* (147 bp) genes produced amplicons that had almost similar product size. Therefore, distinct separation of these amplicons by conventional duplex PCR was not possible. To develop a duplex PCR, duplex real-time PCR using SYBR green was performed using *mprA* (162 bp) and *zmpA* based on the melting curve analysis of amplified products.

In conclusion, the developed PCR assay will be useful for detection and differentiation of *B. pseudomallei* and *B. cepacia*. The combination of *groEL* and *mprA* detection can be used as a confirmatory diagnostic tool for melioidosis, whereas detection of *groEL* and *zmpA* is useful for identification of *B. cepacia*. In addition, developed duplex real-time PCR assay using SYBR green is useful for identification of both *B. pseudomallei* and *B. cepacia* in a single step.

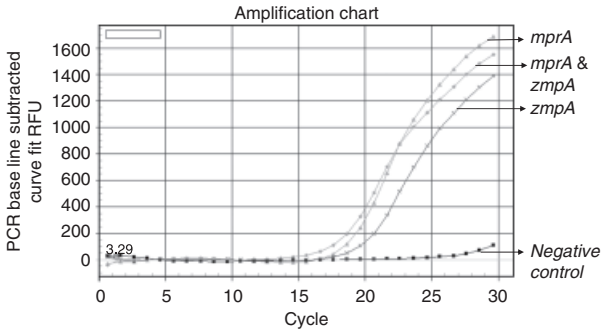

Fig. 5. Amplification curve of *mprA* and *zmpA* genes based on the SYBR green PCR. All the positive controls were amplified between the C_t values 16 and 19.

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