One-step species-specific high resolution melting analysis for nosocomial bacteria detection

Yeng Pooi Wong a,1, Kek Heng Chua b, Kwai Lin Thong a,*,1

a Microbiology Unit, Institute of Biological Science, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
b Department of Biomedical Science, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

ARTICLE INFO

Article history:
Received 4 June 2014
Received in revised form 9 September 2014
Accepted 2 October 2014
Available online 13 October 2014

Keywords:
Nosocomial infections
High resolution melting
Rapid detection
One-step
Sensitive

Abstract
Nosocomial infections are a major public health concern worldwide. Early and accurate identification of nosocomial pathogens which are often multidrug resistant is crucial for prompt treatment. Hence, an alternative real-time polymerase chain reaction coupled with high resolution melting-curve analysis (HRMA) was developed for identification of five nosocomial bacteria. This assay targets species-specific regions of each nosocomial bacteria and produced five distinct melt curves with each representing a particular bacterial species. The melting curves were characterized by peaks of 78.8 ± 0.2 °C for Acinetobacter baumannii, 82.7 ± 0.2 °C for Escherichia coli, 86.3 ± 0.3 °C for Klebsiella pneumoniae, 88.8 ± 0.2 °C for Pseudomonas aeruginosa and 74.6 ± 0.2 °C for methicillin-resistant Staphylococcus aureus. The assay was able to specifically detect the five bacterial species with an overall detection limit of 2 × 10−3 ng/μL. In conclusion, the HRM assay developed is a simple and rapid method for identification of the selected nosocomial pathogens.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Nosocomial infections or health care-associated infections (HAI) are a major public health concern worldwide. The rapid spread and increasing number of HAI remain a medical problem in many hospitals of both developed and developing countries. The prevalence of HAI in mixed patient populations was estimated at 7.6% in high-income countries and 15.5% in low- and middle-income countries (World Health Organisation, 2011). HAI is associated with increased morbidity, mortality, longer length of stay in hospital as well as escalation of health care costs. Infection due to medical care was associated with 9.58 extra days, $38,656 in excess charges, and 4.31% attributable mortality (Zhan and Miller, 2003).

Most nosocomial infections are caused by Staphylococcus aureus, Escherichia coli, Acinetobacter spp., Pseudomonas spp., enterococci and other Enterobacteriaceae (World Health Organisation, 2011). Methicillin-resistant S. aureus (MRSA) is currently the leading cause of HAI. In 2011 alone, the Center for Disease Control and Prevention (CDC) has estimated 80,461 invasive MRSA infections and 11,285 related deaths have occurred (Center for Disease Control and Prevention, 2013). Infections due to Acinetobacter baumannii are usually confined to healthcare settings where its outbreaks typically occur in ICU. Increasing antimicrobial resistance among isolates has been documented and multidrug-resistant A. baumannii is recognized to be among the most difficult antimicrobial-resistant gram negative bacterium to treat and control (Ellipoupolos et al., 2008). Pseudomonas aeruginosa is a versatile opportunist that can infect virtually all tissues but it is most prominent in causing bacteremia in severely burnt patients, chronic lung infection in cystic fibrosis patients and acute ulcerative keratitis among contact lens users (Lyczak et al., 2000). Every year, more than 13% of the estimated 51,000 healthcare-associated P. aeruginosa that cause infections in the US are multidrug-resistant (Center for Disease Control and Prevention, 2013). Both Enterobacteriaceae, namely Klebsiella pneumoniae and E. coli, pose a major health concern as they remain the major extended-spectrum β-lactamase (ESBL)-producing organisms isolated worldwide (Pitout and Laupland, 2008). Estimated 57% of the patients were more likely to die from bacteremia caused by ESBL-producing Enterobacteriaceae compared to those by a non ESBL-producing Enterobacteriaceae (Center for Disease Control and Prevention, 2013).

Early detection of the causative agent of HAI is crucial for effective treatment. Accurate identification of nosocomial pathogens is important to initiate an antimicrobial therapy and better prognosis for patients (Harbarth et al., 2003). It is therefore essential to develop a rapid identification for the causative pathogens that can help in antibiotic selection for treatment and reduces morbidity and mortality. In general, the standard approach for microbial detection in the laboratory from culture incubation to antimicrobial sensitivity testing will take about 2–4 days to complete. Long turnaround times may be a hindrance.
to deliver appropriate therapy to patient. Therefore, to date many molecular based diagnostic techniques have been actively established to detect these pathogens but it was either for a single pathogen (Patel et al., 2011) or broad ranged (Lehmann et al., 2008). A multiplex conventional polymerase chain reaction (PCR) method to detect 5 different nosocomial pathogens was previously developed (Thong et al., 2011). Similar to other conventional PCR approaches, this method requires manual post PCR processing which is time consuming and laborious. Another report of detection of nosocomial pathogens by real-time PCR uses old generation dyes and required two separate reactions (Anbazhagan et al., 2011).

The study of DNA structure and composition using melting or denaturation of double stranded DNA has been used for many years. However, recent technological advances have improved its sensitivity and specificity. The development of saturating DNA dyes and better instrumentation to measure melting behavior have improved its temperature precision and increased measurements per time unit (Vossen et al., 2009). This new post-PCR analysis method has been named as high resolution melting (HRM) analysis. It is a relatively new, simple, inexpensive, and rapid method (Wittwer, 2009).

The use of real time PCR coupled with HRM assay has several advantages over conventional PCR. First, it can bypass the need to prepare gels and use of hazardous chemicals. It is also more rapid as melting is faster than electrophoresis (Vossen et al., 2009). Direct characterization of amplicons is done in a closed system with reduced risk of contamination. Data analysis is performed automatically and the information obtained is quantitative (Ngui et al., 2012). Since this method is non-destructive, amplicons can still be analyzed on a gel if ambiguous result is obtained (Vossen et al., 2009).

HRM analysis is useful for genotyping, mutation detection and also for microorganism species identification. It has been an established fact that HRM analysis offers a low cost and straight forward method when compared to other species identification methods like gene sequencing, mass spectrometry and species-specific hybridization probes. Many broad-range based bacterial identification by HRM have been developed (Cheng et al., 2006; Ozbak et al., 2012) and one particular study can detect up to 100 clinically relevant bacteria by targeting 16S rRNA gene (Yang et al., 2009). While all of these studies were able to detect nosocomial bacteria, most of the published approaches required further steps for definitive identification and none of them employs a multiplex technique. One-step bacterial identification using species-specific targets on this platform has never been attempted. Hence, the main objective of this research was to develop a HRM assay for differentiation of selected nosocomial pathogens namely A. baumannii, E. coli, K. pneumoniae, P. aeruginosa, and MRSA by using species-specific targets in a single step.

2. Materials and methods

2.1. Bacterial isolates

Five types of known nosocomial bacteria were used in the development of the assay, namely A. baumannii (n = 3), E. coli (n = 3), K. pneumoniae (n = 3), MRSA (n = 3), and P. aeruginosa (n = 3). For specificity evaluation, another 150 known bacterial strains which included A. baumannii (n = 12), E. coli (n = 23), K. pneumoniae (n = 17), MRSA (n = 22), P. aeruginosa (n = 19), methicillin sensitive S. aureus (MSSA) (n = 2), Citrobacter spp. (n = 4), Aeromonas hydrophila (n = 1), Morganella morganii (n = 3), Providencia rettgeri (n = 3), Vibrio cholerae (n = 16), Salmonella enterica serovar Typhimurium (n = 20), Salmonella enterica serovar Typhi (n = 3), and Listeria monocytogenes (n = 5) were used. These bacteria were obtained from the stock culture collection of the laboratory. All the five types of nosocomial bacteria were previously isolated from inpatients admitted to local tertiary hospitals. The rest were mixture of clinical and zoonotic origins.

2.2. DNA extraction and quantitation

Bacterial genomic DNA was extracted with the Wizard® Genomic DNA Purification Kit (Promega). These genomic DNA was then measured using a Nanodrop 2000 (Thermo Scientific, USA) to determine the purity and concentration. DNA was then stored at −20 °C for use in further experiments.

2.3. Primer design

Primers for HRM analysis were designed according to the specifications given for best result when conducting a HRM experiment where the length of amplicons should be less than 250 base pairs and the primers itself should be around 20 base pairs with GC content of 30–80%. Optimal annealing temperature of the primers is 59 °C (58–60 °C). Five pairs of primers targeting species-specific genes as listed in Table 1 were designed using online Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) where the specifications stated above were included in the design.

2.4. Conventional PCR

Identities of the five selected organisms were confirmed using optimized published method (Thong et al., 2011). Briefly, the PCR was carried out in a total volume of 25 μL containing 100 ng of DNA template, 1X of buffer, 0.3 μM of each GltA, PhoA, OprL, and Mdh primers, 0.5 μM of MecA primer, 0.4 μM of femA primer, 200 μM of dNTP mix, 1.5 mM of MgCl2, and 1 U of Taq DNA polymerase (Promega, USA). The PCR mixture was then subjected to denaturation at 95 °C for 5 min, followed by 30 cycles of 96 °C for 1 min, 52 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were resolved on a 2.0% (w/v) agarose gel stained with GelRed™ (Biotium Inc, CA, USA), visualized under UV, and analyzed using a Gel Doc system (BioRad, CA, USA).

2.5. Real-time PCR-HRM analysis

The reaction mixture for PCR-HRM with a final volume of 20 μL contained 20 ng of genomic DNA, 0.3 μM of each primer, and 10 μL of 2X MeltDoctor™ HRM Master Mix (Applied Biosystem Inc, USA). Real time PCR and HRM were carried out in a 7500 Fast Real-time PCR system (Applied Biosystem Inc, USA). The cycling parameters were set according to the optimized protocol where enzyme activation was first carried out at 95 °C for 10 min, followed by 40 cycles of amplification consisting of 95 °C for 15 s (denaturation step), and 60 °C for 1 min (primer annealing and elongation step). In the same real-time PCR machine, the amplicons were then continued with melting step to dissociate the double strand. The process was set as denaturation at 95 °C for 10 s, annealing at 60 °C for 1 min, 95 °C for 15 s (high resolution melting), and final annealing at 60 °C for 15 s. The PCR amplicons were denatured and reannealed and the system will record the change in fluorescence (melting) for the particular amplicon. The recorded melting curve profiles were then analyzed using HRM analysis software for Windows® version 3.0.1.

2.6. Sensitivity and specificity

Sensitivity evaluation of the newly developed detection assay was carried out by running the assay using a 10 fold serial dilution of the genomic DNA of each type of bacteria from the concentration of 20 ng/μL. Specificity of the detection method was evaluated using genomic DNA obtained from different types of bacteria.
3. Results

3.1. Real-time PCR-HRM analysis

After bacterial identity confirmation by respective identification PCR (results not shown), the genomic DNA was then tested using real-time PCR coupled with HRM analysis. Amplification of genomic DNA of the A. baumannii, E. coli, K. pneumoniae, P. aeruginosa, and MRSA yielded five different melting curves (Fig. 1). The results obtained were reproducible when the experiments were repeated three times. The derivative melt curves produced by HRM software showed 5 distinct peaks with each representing one particular type of bacteria whereby the peak of 78.8 ± 0.2 °C represents A. baumannii, 82.7 ± 0.2 °C for E. coli, 86.3 ± 0.2 °C for K. pneumoniae, 88.8 ± 0.2 °C for P. aeruginosa, and 74.6 ± 0.2 °C for MRSA. Similarly, each of them has a different melting temperature as shown clearly in the aligned melt curves (Fig. 2).

3.2. Specificity and sensitivity

The newly developed HRM assay was then subjected to a blind test using 150 bacterial strains. The results showed that this assay has 100% specificity in which the 12 A. baumannii, 23 E. coli, 17 K. pneumoniae, 19 P. aeruginosa, 22 MRSA yielded positive results and were correctly identified, while negative results were obtained for the rest of 57 strains. Positive samples were identified by the presence and correct position of peak shown in the derivative melt curves. On the other hand, the detection limit was found to be 2 × 10^{-2} ng/μL for MRSA and K. pneumoniae while 2 × 10^{-3} ng/μL was found for the other three types of nosocomial bacteria.

4. Discussion

Ideally, one pair of primers should be used in HRM to detect a particular unknown nosocomial bacterium. Therefore, most previously

### Table 1

List of primers sets used in the real-time PCR coupled with HRM analysis and its properties.

<table>
<thead>
<tr>
<th>Nosocomial pathogens</th>
<th>Target gene</th>
<th>Primer pairs (5′➔3′)</th>
<th>Amplicon size (bp)</th>
<th>GC content of amplicon (%)</th>
</tr>
</thead>
</table>
| Acinetobacter baumannii | gltA | F1: GTGGCACATTAGGTCCCGA  
R1: CAAGGTAGTCTGCTTGAGTCG | 189 | 43.9 |
| Escherichia coli | uidA | F2: CATACCTGTTCACCGACGAC  
R2: CTGCCAGGAGAAACTGCATC | 174 | 53.4 |
| Klebsiella pneumoniae | khe | F3: GGGAGGTTTACGTCTCAAC  
R3: GTACTTCTTGTTGGCCTCG | 272 | 60.7 |
| Pseudomonas aeruginosa | oprL | F4: ATTECGGAAATTTGCGC  
R4: GGAGCTGTCGTACTCGAGT | 209 | 63.2 |
| MRSA | mecA | F5: GGCAGACAAATTGGTTGTT  
R5: TCAGAGCACCACGCTTGCG | 214 | 33.6 |

![Derivative Melt Curves](image)
reported bacteria detection methods using HRM analyses were focused on genus identification targeting highly conserved gene (Cheng et al., 2006; Yang et al., 2009). In our initial development and optimization of HRM assay, we targeted the 23S rRNA to amplify the five selected bacteria. However, it failed to work due to the overlapping Tm. Consequently, plans for specific primers targeting each bacterial species were undertaken. Although incorporating five pairs of primers into this system may be difficult, it is still possible to do so as other studies have tried up to a tetraplex format using the HRM analysis (Bidet et al., 2012; Slinger et al., 2011; Zeinzinger et al., 2012). Indeed, careful primer design was required in order to minimize unspecific bands and at the same time to avoid overlapping Tm. Hence, our design of the species-specific primers was therefore directed toward the GC content of the amplicons, which is the main factor that affects its Tm (Table 1).

The choice of targets was also very important in the development of this detection assay. Genes selected for amplification should be present all the time in the particular pathogen. Overall, the mecA gene was selected for identifying MRSA as it is the gene that encodes for penicillin-binding protein, PBP2a which is the structural determinant for methicillin resistance in staphylococci (Chambers, 1997). Although the choice of mecA as a target may potentially miss the detection of some isolates that carry the recently found mecC variant, prevalence of these variants is still considered low and mecA variants still represent the majority of MRSA (Paterson et al., 2014a,b). As for A. baumannii, g1IA gene that encodes for a citrate synthase was chosen to be the target as it is one of the common housekeeping genes for this particular bacterium (Bartual et al., 2005). The aprl gene was reported to be conserved in P. aeruginosa so it has been selected as our target (De Vos et al., 1997). The uidA gene in E. coli was used as it is commonly reported for E. coli identification (Chandra et al., 2013; Monday et al., 2001; Muller et al., 2007). The hemolysin gene, khe, present in the chromosome of K. pneumoniae is a unique and highly conserved gene which should be a good choice for this platform (Hartman et al., 2009; Yin-Ching et al., 2002).

In this study, we demonstrated a simple, sensitive and one-step approach for rapid identification of nosocomial bacteria. Third generation saturating dye, such as SYTO®9 that was used in this assay gave us a more confident result as compared to the first generation dyes like SYBR Green 1 (Anbazhagan et al., 2011). Third generation saturating dye is a better choice for real-time PCR applications in terms of reproducibility because concentration of this dye affects amplipon Tm less extremely, so broader range of dye concentrations can be used without causing PCR inhibition to produce a more consistently shaped melt curve (Monis et al., 2005). The use of specific targets minimizes the chances of misidentification of closely related species that was faced by one of the broad-range PCR coupled with HRM analysis approach (Cheng et al., 2006). However, the broad-ranged PCR-HRM method also require further steps to confirm bacterial identity of many clinically important species such as A. baumannii, E. coli and K. pneumoniae after the initial HRM analysis (Cheng et al., 2006). This was achieved by either heteroduplex formation of the PCR amplicon of tested and reference bacteria or second PCR targeting different 16S rRNA region which was unnecessary in our approach. Moreover, this approach also bypassed the need for lengthy coding assignment based on subsets and difference plots to cross reference with their standard curves (Yang et al., 2009). The versatility of this approach is that we can customize it to be either a single, double or multi-pathogen detection depending on the need by altering the composition of the primer sets. Furthermore, this approach has superior sensitivity and comparable specificity to the previously reported method (Thong et al., 2011). In times of critical serious infections, which are often for HAI, this approach can serve as an alternative method for preliminary diagnosis in order to facilitate and hasten the delivery of proper antimicrobial treatment.

However, this approach can never replace the role of antimicrobial susceptibility testing and conventional culture based identification. As this method is for nosocomial species detection, it does not provide information on pathotypes, genotypes, and microbial resistance; apart from knowledge of methicillin resistance in S. aureus (Chambers, 1997). Other methods like mass spectrometry that can detect antimicrobial resistance are still dependent on culture enrichment and cost may also be a limiting factor (Hrabak et al., 2012; Sparbier et al., 2012; Slinger et al., 2011; Zeinzinger et al., 2012). Indeed, careful primer design was required in order to minimize unspecific bands and at the same time to avoid overlapping Tm. Hence, our design of the species-specific primers was therefore directed toward the GC content of the amplicons, which is the main factor that affects its Tm (Table 1).
Since the targets were designed specifically for the five nosocomial bacteria, negative results will have to rely on other methods for confirmation.

In conclusion, this study shows that a simultaneous detection of the selected nosocomial bacteria using real time PCR-HRM analysis is possible. This approach offers a simple one-step method that is rapid and sensitive that can be applicable in diagnosis of HAI in clinical laboratory.

Financial support

The work was financially supported by High Impact Research Grant (UM.C/625/HIR/MOHE/02) and GA 03-2013 (Molecular Diagnostics of Bacteria). Yung Pooi Wong was supported by a fellowship from the Ministry of Health, Malaysia.

Disclaimer

No competing interests exist.

Acknowledgment

We thank University of Malaya for support and facilities. We also thank Boon Pin Kee and Soo Tein Ngoi for the technical guidance.

References


