Multiplex PCR Detection of Five Common Respiratory Pathogens from Bronchoalveolar Lavages Using High Resolution Melting Curve Analysis

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Abstract

Background

The study describes the application of the Multiplex High Resolution Melting curve (MHRM) assay for the simultaneous detection of five common bacterial pathogens (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Escherichia coli*) directly from bronchoalveolar lavage samples.

Results

Our MHRM assay successfully identified all five respiratory pathogens in less than 5 hours, with five separate melting curves with specific melt peak temperatures (T_m). The different T_m were characterized by peaks of $78.1 \pm 0.4^\circ\text{C}$ for *S. aureus*, $83.3 \pm 0.1^\circ\text{C}$ for *A. baumannii*, $86.7 \pm 0.2^\circ\text{C}$ for *E. coli*, $90.5 \pm 0.1^\circ\text{C}$ for *K. pneumoniae*, $94.5 \pm 0.2^\circ\text{C}$ for *P. aeruginosa*. The overall sensitivity and specificity of HRM were 100% and 88.8%, respectively.

Conclusions

Our MHRM assay offers a simple and fast alternative to culture approach for simultaneous detection of five major bacterial Lower respiratory tract infection pathogens. Utilization of this assay can help clinicians initiate prompt and appropriate antimicrobial treatment, towards reducing the morbidity and mortality of severe respiratory infections.

Background

Lower respiratory tract infections (LRTIs) are the main cause of hospitalization and death around the world, responsible for 2.38 million deaths annually [1, 2]. Nosocomial pneumonia or hospital acquired pneumonia (HAP) is a major health problem in many hospitals of both developed and developing countries [3]. HAP is most often caused by both Gram-positive and Gram-negative bacteria. The most common bacteria implicated in the development of HAP are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *klebsiella pneumoniae*, *Acinetobacter baumannii* and *Escherichia coli* [4]. These pathogens are often resistant to various antibiotics, making LTRIs caused by them difficult to treat [5, 6]. It is clear that a fast, simple and accurate diagnosis for the detection of LRTI pathogens is vital to the selection of antibiotic therapy and management of patient treatment [7]. The current standard for LRTIs is bronchoalveolar lavage (BAL) culture, but this is time-consuming, labor-intensive and has low sensitivity, particularly when the patient has been given antibiotic therapy prior to sampling [7–9]. For these reasons, molecular methods have been developed to improve the diagnosis of bacterial respiratory infections. One of these methods is high-resolution melting (HRM) analysis, which relies on a real-time PCR method in the presence of the double stranded DNA (dsDNA) intercalating fluorescent dye and monitors changes in melting of dsDNA with increasing temperature [10–14]. HRM has been used for genotyping, detection of bacterial resistance genes, as well as for detection and differentiation of various pathogenic organisms such as bacteria, fungi, viruses and parasites [11, 15–21]. However, this method has not been applied directly on BAL samples to simultaneously distinguish several pathogens. In this study we have developed a single-round Multiplex HRM (MHRM) assay for the simultaneous detection of five common bacterial pathogens (*P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *A. baumannii* and *E. coli*) directly from BAL samples.

Results

HRM speciation assay

Our results indicate that the HRM assay designed in this study was able to distinguish all the five standard bacterial species. Fig 1 shows the unique pattern of the derivative melting curve, aligned melt curves, and differentiation plots, which demonstrates a unique T_m for each bacterial species that helps differentiate all five test bacterial species in a single HRM reaction. The melting curves were characterized by peaks of $78.1 \pm 0.4^\circ\text{C}$ for *S. aureus*, $83.3 \pm 0.1^\circ\text{C}$ for *A. baumannii*, $86.7 \pm 0.2^\circ\text{C}$ for *E. coli*, $90.5 \pm 0.1^\circ\text{C}$ for *K. pneumoniae*, $94.5 \pm 0.2^\circ\text{C}$ for *P. aeruginosa*. The overall T_m ranges with each of the pathogens are illustrated in Fig 2.

MHRM Limit of Detection and Specificity

The limit of detection (LOD) of MHRM assay for *A. baumannii*, *P. aeruginosa*, *K. pneumoniae* and *E. coli* was between $0.8-1 \times 10^3$ CFU/ml, and for *S. aureus*, it was 1.3×10^3 CFU/ml. Data revealed that MHRM assay specifically detected all 5 test pathogens, without non-specific amplifications, when DNA from human cells or other species of bacteria was used as template.

Comparison of MHRM assay to culture identification of pathogens

Of 96 BAL specimens analyzed, 35 specimens (36.4%) were concordantly negative by culture as well as MHRM. The detection rate of MHRM was higher than that of culture, since 54 (56%) of samples were culture positive, whereas 61 samples (63.5%) were positive by MHRM assay ($P < 0.001$). Table 2 compares the list of identified pathogens recovered by both culture and MHRM, and demonstrates that all culture-positive samples were also MHRM positive, with identical species identification (Table 2). *A. baumannii* (23%, 22/96) was the most common pathogen, as determined by both culture and MHRM assay. In five samples out of 96, two melting peaks were observed in the MHRM derivative plots, suggesting the presence of two pathogens (three samples: *E. coli*+ *A. baumannii* and two samples: *P. aeruginosa*+ *K. pneumoniae*), but culture identified one pathogen (Table 3). Two samples out of 96, showed three melting peaks in the HRM derivative plots, suggesting the presence of three pathogens (one sample: *A. baumannii*+ *P. aeruginosa* + *K. pneumoniae* and one sample: *A. baumannii* + *K. pneumoniae*+ *S. aureus*) but culture identified one and two pathogens for each sample, respectively (Table 3). Compared to culture, the specificity of the MHRM ranged from 88.8% to 100%, and sensitivity 100% for all test pathogens (Table 3).

Discussion

The rapid and accurate identification of LRTIs are critical for appropriate antimicrobial therapy, which is strongly associated with positive clinical outcomes [7, 8]. Various molecular methods have been used for diagnosis of LRTIs; these include RT-PCR, MALDI-TOF MS, BIOFIRE Film Array system and DNA sequencing [7, 22]. However, most of these methods are expensive, time consuming, and technically challenging. In this study, a MHRM assay has been successfully developed for rapid and accurate identification of five common respiratory bacterial pathogens directly from BAL specimens. Our results indicate that MHRM assay is highly specific as all targets were identified accurately, with no fluorescence signal detected in samples containing non-target DNA. The melting temperature for DNA from each target pathogen was sufficiently different (ideally $>1^\circ\text{C}$), to enable simultaneous discrimination between the all pathogens in BAL sample. The melting profile of unknown samples was correctly matched with melting profiles of standards with known DNA, and the results were obtained within less than 5 hours in comparison with biochemical or culture-based methods, for which results are available for 24-48 h. Quantification of the bacteria in the LRTIs is the key to differentiate between contamination and true infection [23]. In this study, the LOD of the MHRM assay ranged between 8×10^2 to 1.3×10^3 CFU/ml for different target pathogens, which is lower than the number of bacteria suggested for identification of bacterial infection via culture (10^4 CFU/ml). Therefore, samples which are reported as culture negative and MHRM positive cases can be explained as contamination or colonization. Some of the discrepant results (HRM-positive but culture-negative) observed in this study lies in the fact that molecular assays can amplify the DNA from dead organisms, resulting in clinically false-positive results [24]. One of the main drawbacks of the HRM assay is its inability to accurately quantify the number of pathogens in samples. Although a linear relationship existed between fluorescence values and bacterial concentration in samples, it is impossible to determine the exact number of bacteria. So, clinicians cannot judge that the sample is from an infection or colonization. Therefore, a positive result by HRM assay should be carefully analyzed, considering clinical symptoms, chest radiograph findings and other laboratory tests, such as CRP and WBC results [25]. Considering the fact that coinfections with a mix of 2-3 bacteria can occur in lower respiratory tract infections [25, 26], and current rapid methods have poor sensitivity for identifying all species in mixed samples, therefore, the ability of the assay for detection of mixed infections is considerably important. The results of mock mixed infections indicated that the HRM assay has the potential to detect poly-microbial infections. However, the results from clinical samples revealed that the sensitivity of the HRM assay in detection of poly-bacterial clinical samples is lower than the sensitivity in detection of mono bacterial infections (Table 2). In this study, the HRM assay showed more than 88% specificity and 100% sensitivity for each pathogen. Another HRM method developed by Edwards et al. reported an overall sensitivity of 97.1% and a specificity of 100% for detection of six common Gram-negative pathogens [11]. This difference may be related to the fact that we extracted DNA directly from the BAL samples, but Edwards et al. was extracted DNA from the pure cultures or single colonies.

Conclusion

We have developed a MHRM assay that may be used as a functional tool for the diagnosis of LRTIs through detection of the potential pathogen directly from the clinical BAL samples. By offering an accurate simultaneous identification of the causative agent(s) of LRTI within a timeframe much shorter than culture method MHRM can help clinicians to initiate timely and appropriate antimicrobial therapy, and hence reduce morbidity and mortality associated with LTR infections.

Materials

Study design and identification of isolates

A total number of 96 BAL specimens were collected from hospitalized patients with suspected pneumonia from four hospitals (Tehran, Iran) during May 2018–January 2019. All specimens were cultured on the 5% sheep blood, chocolate, MacConkey agar plates and incubated at 37°C overnight. Bacterial colony counts and species identification were carried out according to validated standard operation procedures [23, 27]. BAL cultures were considered positive if 10^4 bacteria or more per milliliter of BAL were found [23].

DNA extraction

DNA was directly extracted from BAL specimens and standard isolates using the FAVOGEN DNA Extraction Kit (Biotech Corp, Taiwan), following the protocol for extraction. Concentration and purity of extracted DNA were determined by a Nanodrop® 2000c instrument (Thermo Fisher Scientific, USA). DNA was kept at -20 °C for future experiments.

Primer designing for the study

The whole genome sequences of *P. aeruginosa* (GenBank accession number CP050332), *K. pneumoniae* (GenBank accession number CP077773), *S. aureus* (GenBank accession number CP053639), *A. baumannii* (GenBank accession number CP000521), and *E. coli* (GenBank accession number CP034658) were downloaded from NCBI database. Comparative analysis of the chromosomes of these five species was performed in order to identify conserved regions (species specific sequences). Five primer sets were designed using an online primer3 software (<http://primer3.ut.ee/>) and the specificity of primers was assessed *in silico* using primer-BLAST program available at <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>. The theoretical T_m of amplicons was calculated with Oligonucleotide Properties Calculator (OligoCalc) based on the amplicon sequence (<http://biotools.nubic.northwestern.edu/OligoCalc.html>). The five primer sets generated 153 to 272 bp products and the sequences of the forward and reverse primer, their targets, and predicted amplicon T_m are presented in Table 1. The efficiency of primers was assessed by using conventional PCR. A set of conventional gradient PCR was performed to ensure that the five pairs of primers were able to amplify the target region in the five species without producing unspecific PCR products or primer dimers that interfere with the interpretation of results in PCR-HRM analysis later on. The reaction mixture contained 12.5µL PCR Master Mix 2X (Ampliqon, Denmark), 0.5 µL of each primer (10 pmol, Metabion, Martinsried, Germany), 1 µL of DNA (10–20 ng/µl) and 6.5 µL of DNase-free water in a total reaction volume of 25 µl per sample. The PCR thermocycling conditions consisted of an initial denaturing step at 95 °C × 50 seconds, followed by 30 cycles of denaturation at 95°C ×15 s, annealing from 56 °C to 65 °C×20 seconds, extension at 72 °C× 20 seconds and a final extension at 72 °C×10 minutes. The reactions were performed in a T100™ thermal cycler (Bio-Rad). The amplified DNA fragments were electrophoresed in a 1.5% agarose gels with 0.5X TBE (Tris/Borate/EDTA) buffer. The DNA bands were visualized by KBC power load dye staining and photographed under UV illumination.

Multiplex HRM-real-time PCR assay

Multiplex Real-time PCR with HRM (MHRM) analysis was performed sequentially on a ABI StepOnePlus Real-Time PCR (Applied Biosystems) in a reaction mixture containing 4 µl of 5x HOT FIREPol® EvaGreen® HRM Mix no ROX (Soils Biodyne, Estonia), 0.5µL of pathogen-specific primer (10 pmol, Metabion, Martinsried, Germany), 1µL of DNA (10–20 ng/µl) and 6.5µL of DNase-free water in a total reaction volume of 20µl per sample. Positive controls (containing genomic DNA from each species) and negative controls (DNase free water) were included in each run. The reaction conditions involved enzyme activation at 95 °C× 12 minutes, followed by 40 cycles of denaturation at 95°C×15 seconds, 63°C×20 seconds for annealing, and 72°C×20 seconds for extension. Following this, HRM was carried out at 95 °C× 15 sec and a melting profile from 60°C to 95°C using a ramping degree of 0.3°C/sec. The melt curve analysis was carried out using HRM Software version 3.0.1(Applied Biosystems).

Limit of detection

The LOD of MHRM assay was determined by 10 fold serial dilutions of 0.5 McFarland suspension of each isolate, ranging from 10^7 to 10^1 CFU/ml. A 10µl sample of each dilution was plated and the colonies were counted to determine the CFU/ml in each dilution. Genomic DNA was extracted from all dilutions by DNA Extraction Kit (Biotech Corp, Taiwan), according to the manufacturer's instructions. The specificity of the HRM assay was tested using various DNA from other organisms such as human genomic DNA; *Staphylococcus epidermidis*, *Salmonella typhimurium*, *Streptococcus pneumoniae*, *Haemophilus influenza*, *Proteus*

mirabilis and *Streptococcus pyogenes*. We calculated the sensitivity and specificity of the real-time PCR with MHRM analysis for the detection of five common bacterial pathogens compared with culture, which was used as the reference standard.

Mock Mixed infections

In order to assess the ability of MHRM to detect mixed infections, extracted DNA from each of the *P. aeruginosa*, *S. aureus*, *K. pneumonia*, *A. baumannii* and *E. coli* were mixed with other DNA of two, or three above mentioned pathogens. Consequently, 1 µl of double, triple and quadruple bacterial DNA combinations were tested by MHRM assay.

Statistical analysis

The sensitivity and specificity of the MHRM assay was determined by comparison to culture, as the reference standard method. Kappa correlation was utilized to assess the degree of agreement between the two methods. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS®) software (version 21, IBM Corp.).

Abbreviations

LRTIs

Lower respiratory tract infections

HAP

hospital acquired pneumonia

Pseudomonas aeruginosa

P. aeruginosa

Staphylococcus aureus

S. aureus

Klebsiella pneumoniae

K. pneumoniae

Acinetobacter baumannii

A. baumannii

Escherichia coli

E. coli

BAL

bronchoalveolar lavage

HRM

high-resolution melting

T_m

melting temperature

dsDNA

double stranded DNA

MHRM

Multiplex HRM

LOD

limit of detection

Declarations

Ethics approval and consent to participate

The study was approved by the ethics committee of Tehran University of Medical Sciences (approval number: IR.TUMS.MEDICINE.REC.1397.459). All methods were carried out in accordance with the relevant guidelines and regulations. No BAL samples were drawn for this study, BAL samples collected as routine clinical care for these patients and remnant of samples were used. Therefore, informed consent was not sought, and informed consent waiver was approved by the ethics committee of Tehran University of Medical Sciences

Consent for publication

Not applicable.

Availability of data and materials

Please contact author for data requests

Competing interests

The authors declare no conflicts of interest

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Authors' contributions

RB designed the experiments. JG conducted the experiments. FJ and ME contributed materials and analysis tools. RB and JG drafted the manuscript. FBH revised the manuscript and advised in all parts of the study. All authors read and approved the final manuscript.

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Tables

Table1. Comparison of sequence, amplicon size, GC content and melting tempreture of specific PCR primers used for each pathogen in the MHRM analysis. List of primers used in this study and its properties.

Pathogen	Gene Bank accession no.	primer sequence/ Tm (°C)	Nucleotide positions	Amplicon size (bp)	GC content of amplicon (%)	Predicted Tm ^a (°C)	Observed Tm ^b (°C)
<i>A. baumannii</i>	CP000521	F: GTGGCACATTAGGTCCCGA (56.4) R: CAAGGTAGTCTGCTTGAGTCG (58.4)	3143607-3143795	189	44	82.09	83.53
<i>K. pneumonia</i>	CP077773	F: GGCGAGGTTTACGTCTCAAC (55.9) R: GTACTTCTTGTTGGCCTCGC (56.2)	5129431-5129702	272	61	89.34	90.29
<i>P. aeruginosa</i>	CP050332	F: ATCTTCTGGCTGTCTTCGGC (55.3) R: AATGTCCACCACGGTCTTCC (56.3)	2396197-2396388	192	70	92.77	93.67
<i>S. aureus</i>	CP053639	F: GCTAAACCACTTTTGTAGCACC (58.7) R: TGATAAAGAAAATGGCATGCACA (57.6)	1867646-1867798	153	31	76.44	78
<i>E. coli</i>	CP034658	F: CATACCTGTTACCGACGAC (55.4) R: CTGGCAGGAGAACTGCATC (56.1)	1662333-1662506	174	53	84.95	86.14

^aAmplicon melt point calculated by OligoCalc program

^bAmplicon melt point calculated by ABI StepOnePlus Real-Time PCR instrument

Table 3. Comparison of sensitivity and specificity of MHRM assay versus culture identification method, and the degree of agreement between the methods for each test pathogen.

Target	True Positive	True Negative	False Positive	False Negative	Sensitivity (%)	Specificity (%)	Agreement (%)	Cohen's Kappa
<i>A. baumannii</i>	22	71	3	0	100	97.3	96.9	0.92
<i>K. pneumonia</i>	17	71	8	0	100	88.8	91.6	0.76
<i>P. aeruginosa</i>	9	85	2	0	100	97.7	97.9	0.89
<i>S. aureus</i>	9	87	0	0	100	100	100	1.00
<i>E. coli</i>	4	90	2	0	100	97.8	92.7	0.79

Figures

Table 2. Comparative analysis of HRM and culture identification			
Infection level of BAL specimens No. (%)	HRM	Culture	Frequency No. (%)
Single bacterial infection N=48 (50%)	<i>A. baumannii</i>	<i>A. baumannii</i>	14 (14.6)
	<i>K. pneumonia</i>	<i>K. pneumonia</i>	12 (12.5)
	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	7 (7.3)
	<i>S. aureus</i>	<i>S. aureus</i>	6 (6.3)
	<i>E. coli</i>	<i>E. coli</i>	2(2.1)
	<i>K. pneumonia</i>	-	4 (4.2)
	<i>A. baumannii</i>	-	2 (2.1)
	<i>P. aeruginosa</i>	-	1 (1)
Double bacterial infection N=11 (11.5%)	<i>A. baumannii</i>	<i>A. baumannii</i>	2(2.1)
	<i>E. coli</i>		
	<i>A. baumannii</i>	<i>A. baumannii</i>	1(1)
	<i>S. aureus</i>	<i>S. aureus</i>	
	<i>A. baumannii</i>	<i>A. baumannii</i>	3(3.1)
	<i>K. pneumonia</i>	<i>K. pneumonia</i>	
	<i>A. baumannii</i>	<i>E. coli</i>	1(1)
	<i>E. coli</i>		
	<i>E. coli</i>	<i>E. coli</i>	1(1)
	<i>K. pneumonia</i>	<i>K. pneumonia</i>	
	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	2(2.1)
	<i>K. pneumonia</i>		
Triple bacterial infection N=2 (2.1%)	<i>K. pneumonia</i>	<i>K. pneumonia</i>	1(1)
	<i>S. aureus</i>	<i>S. aureus</i>	
	<i>A. baumannii</i>	<i>A. baumannii</i>	1 (1)
	<i>K. pneumonia</i>	<i>S. aureus</i>	
	<i>S. aureus</i>		
Negative specimens N=35 (36.4%)	None	None	35(36.4)

Figure 1

Discrimination of five different species targeted by high resolution melting (HRM) analysis. Each species indicated a unique melting peak. **(A)** Derivative melt curves, **(B)** Aligned melt curves **(C)** difference plot of various bacteria. The same colors illustrate the same pathogens. Blue curve (*S. aureus*); orange curve (*E. coli*); Green curve (*A. baumannii*); gray curve (*K. pneumoniae*); yellow curve (*P. aeruginosa*).

Figure 2

Box plot showing the variation ranges of melting temperature of each pathogen in clinical samples. The ANOVA test showed significant difference of average T_m values between pathogens ($P=0.001$). Sa (*S. aureus*), Ab (*A. baumannii*), Ec (*E. coli*), Kp (*K. pneumoniae*), Pa (*P. aeruginosa*).