original article

A novel high-resolution melting analysis approach for rapid detection of vancomycin-resistant enterococci

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BACKGROUND: Vancomycin-resistant enterococci (VRE) are resistant to most classes of antibiotics. Diagnosis of VRE using standard methods takes 2 to 5 days. Development of a rapid PCR-assay that detects and identifies resistant genes in bacteria would provide time-critical information on the presence of VRE in clinical samples allowing early treatment and management of infected patients.

OBJECTIVES: Investigate the use of high resolution melting analysis (HRMA) and 16S-rRNA-PCR approach for rapid and cost-effective identification of VRE.

DESIGN: Descriptive antibiotic susceptibility studies.

SETTING: Manchester Academic Health Sciences Centre and School of Translational Medicine, University of Manchester, UK, and Department of Clinical Laboratory Sciences, Taibah University, Saudi Arabia.

MATERIALS AND METHODS: PCR-HRMA using 16S-rRNA V1-primers was used to detect and identify VRE. DNA from different strains of vancomycin-resistant and -sensitive *Enterococcus faecalis* (VSE) and *Enterococcus faecium* were amplified using V1-primer followed by HRMA in a single run. Differentiation of VRE from VSE was based on curve shapes generated against reference organisms (*Bacteroides fragilis*).

MAIN OUTCOMES MEASURES: Amplification curves and difference plots for VRE and VSE.

RESULTS: Difference plots were generated for all vancomycin-resistant and -sensitive *E faecalis* and *E faecium* strains by subtracting their fluorescence melting profile from that of a reference-species *B fragilis*. A characteristic curve shape was produced by vancomycin-sensitive *E faecalis* and *E faecium*. However, vancomycin-resistant strains of these bacteria were associated with a markedly different curve shape facilitating a clear differentiation.

CONCLUSION: The 16S-PCR-HRMA approach has the potential for detecting vancomycin-resistant *E faecium* and *E faecalis*. Data with VRE provide the basis for combining VRE identification with pathogens speciation in a rapid, cheap assay able to identify a pathogen as an *Enterococcus* and whether it is vancomycin-sensitive or -resistant *E faecium* or *E faecalis* in a single PCR and HRMA run.

LIMITATIONS: Tested on specific, but not all, reference *Enterococcus* species and clinical isolates.

CONFLICT OF INTEREST: None.

Choosing an appropriate antimicrobial therapy for critically ill patients is a major problem for healthcare providers due to the increasing prevalence of drug-resistant microorganisms in the hospital environment and particularly in critical care.[1] Treatment and management of patients infected by resistant strains has become more difficult than those infected by susceptible organisms.[1-3] Infection caused by resistant organisms increases length of stay and mortality rate, and requires use of more toxic drugs and thus increases overall healthcare costs.[3,4] Therefore, the need for clinicians to treat patients with suspected infection as soon as possible means that broad-spectrum antimicrobials are administered until infection is confirmed and the results of an antimicrobial susceptibility tests are available. Early detection and identification of antibiotic-resistant organisms will allow early and optimum treatment and management of the infected patient, and therefore decrease overall healthcare costs.[4] The use of broad-spectrum antimicrobials in this way is one possible reason for the increase in antibiotic-resistant bacteria. One of most common antibiotic-resistant bacteria is vancomycin-resistant enterococci (VRE).[1,5]

The emergence of VRE, particularly *Enterococcus faecalis* and *Enterococcus faecium* has become a significant issue in the control of hospital acquired infections worldwide.[2,5] A high level vancomycin resistance is usually linked to acquisition of the transmissible genetic elements *vanA* or *vanB*, although less common *van* variants such as vanC have also been identified.[2] The vanC gene is usually found in *E gallinarum* and *E casseliflavus/flavescens* as these strains naturally have a low-level of resistance to vancomycin.[2] VRE are resistant to most major classes of antimicrobial therapy and may transfer the vancomycin resistance gene to other bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA).[6,7] Inappropriate antibiotic use appears to be the key driver for VRE emergence while longer hospital stay, and increased colonisation are important in increasing the prevalence of VRE infection.[5,8] Studies about prevalence and outcomes of infection in ICUs worldwide indicate that about 5% of all confirmed infections in ICU worldwide are caused by VRE.[9,10] The high morbidity and mortality and increased costs of care associated with VRE means that early detection is crucial to facilitate optimal treatment and management of the infected patient and ensure they are quickly isolated to reduce the risk of onward transmission.[2,5,6] Laboratory diagnosis of VRE infection using standard culture and antibiotic susceptibility testing is a time-consuming process which can take 2 to 5 days to complete.[2,5] Therefore, there is an urgent need to develop rapid and cost-effective molecular assays that can provide time-critical information on the presence of VRE in clinical samples. Molecular approaches using real-time PCR have been developed to provide results in less time by detection and identification of mainly the resistance genes in bacteria. The development of molecular methods that could rapidly detect and identify pathogens and determine their likely antimicrobial susceptibility would be helpful in the early treatment and management of patients with infection. PCR-based approaches using multiple primers and labelled probes have been applied to detection of *van* genes including commercial assays aimed primarily at screening of faecal or rectal samples.[11,12] High resolution melting analysis (HRMA), a technique for characterising PCR amplicons based on differences in melting profile (curve shape), identifies a wide spectrum of clinically important pathogens from clinical isolates and broth culture.[13,14] The aim of the present study was to investigate the use of HRMA combined with broad range 16S rRNA PCR as an alternative approach for the rapid and cost-effective differentiation of vancomycin-sensitive and -resistant Enterococci strains.

MATERIALS AND METHODS

After developing the PCR-HRMA approach with a multi-decision strategy for detection and identification of 21 common bloodstream pathogens as described by Ozbak and co-workers in 2012,[15] the PCR-HRMA approach was consequently tested for its ability for detecting and identifying VRE, one of most common antibiotic-resistant organisms in bloodstream infections. We used different primer sets from the primers used by Ozbak and colleagues, so as to produce a package that would be useful in routine molecular microbiology laboratory for rapid identification of the most common bloodstream pathogens and detection of resistance from clinical blood samples. Other experiments were carried out to confirm the presence of standard vancomycin-resistant *Enterococcus* strains by detecting their *van* genes and determining whether *vanA* or *vanB*.

This study was done at Manchester Academic Health Sciences Centre and School of Translational Medicine (University of Manchester, UK), and at the Department of Clinical Laboratory Sciences (Taibah University, Saudi Arabia) to investigate the use of HRMA and the 16S-rRNA-PCR approach for rapid and cost-effective identification of VRE. PCR-HRMA using 16S-rRNA V1-primers[16] (**Table 1**) were used to detect and identify VRE. This study was conducted on five reference strains that belong to two groups: vancomycin-resistant *E faecalis* and *E faecium* strains (*E faecium* ATCC 19434

Table 1. Information and sequence data for V1, *vanA* and *vanB* primer pairs.

Primer Pair (PP)		Sequence (5´-3´)	Purification	Melting temperature (°C)
V1	F	GYGGCGNACGGGTGAGTAA	HPLC	58.0
	R	TTACCCCACCAACTAGC	HPLC	60.5
vanA	F	TATGATGGCCGCTGCAGGTA	HPLC	55.8
	R	CGGTGAAATTATCCCAAGTGGC	HPLC	58.2
vanB	F	GCCATGCAAAACCGGGAAAG	HPLC	59.6
	R	CAAGCGATTTCGGGCTGTGA	HPLC	60.1

(VRE), *E faecalis* ATCC 29212 (VRE) and *E faecalis* ATCC 51299 (VRE)), and vancomycin-sensitive *E faecalis* and *E faecium* strains (*E faecalis* NCTC 77 (VSE), *E faecium* NCIMB 2699 (VSE)).

Source of bacterial strains

Reference strains for the two groups included in this study; vancomycin-resistant *Enterococcus* (VRE) strains and vancomycin-sensitive *Enterococcus* (VSE) strains, and the standard strain of *Bacteroides fragilis* were purchased from Pro-Lab (Neston, South Wirral, Cheshire, UK), NCIMB Limited or the National Collection of Type Cultures (NCTC; Health Protection Agency, Porton Down, UK). These referenced bacterial strains were as follow: *Bacteroides fragilis* ATCC 25285, VSE strains (E. faecalis NCTC 77, *E faecium* NCIMB 2699) and VRE strains (*E faecium* ATCC 19434, *E faecalis* ATCC 29212, *E faecalis* ATCC 51299).

Culture and DNA extraction

All strains used in this study were grown under aerobic conditions except *B fragilis* ATCC 25285, which was grown under anaerobic condition at 37°C in 10 mL lysogeny broth (LB) (containing 10 g/L bacto-tryptone, 5 g/L yeast extract 10g/L NaCl) and were then subcultured overnight on blood agar (containing based on SIFMA-ALDRICH 10 g/L meat extract, 10 g/L peptone, 10 g/L sodium chloride and 5 g/L agar) under the appropriate conditions as mentioned previously. Cultures were centrifuged at 3000*g* for 10 minutes and DNA was extracted from the bacterial pellet using High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's protocol. DNA extracts were aliquoted into sterile microtubes and stored at -20°C (*https://lifescience.roche.com/documents/High-Pure-PCR-Template-Preparation-Kit.pdf*).

PCR confirmation of vanA/vanB *expression*

To confirm the expression of *van* genes (for identification of VRE) associated with VRE standards strains used in this study, extracted DNA was amplified and analysed by real-time PCR on a LightCycler 480 using previously published gene-specific *vanA* (Forward TATGATGGCCGCTGCAGGTA; Reverse CGGTGAAATTATCCCAAGTGGC) and *vanB* (Forward GCCATGCAAAACCGGGAAAG; Reverse CAAGCGATTTCGGGCTGTGA) primer sets for detection of *van* genes[17] (**Table 1**) in a 20 µL reaction volume also containing DNA extract (250 fg-250 ng), 0.3 µM forward and reverse primers, $MgCl_2$ at a final concentration of 3 µM and 10 µL (1X) LightCycler 480 High Resolution Melting Master mix. Negative controls containing PCR grade water instead of bacterial DNA extract were included. *vanA* and *vanB* primer sets were optimised to run in the following PCR conditions: activation step at 95°C for 10 minutes followed by 30 cycles of 95°C for 10 seconds, 55°C for 10 seconds and 72°C for 10 seconds. Reactions containing nuclease-free water were also run as negative controls. Each experiment was performed at least three times.

Real-time PCR for high resolution melting analysis

DNA amplification was performed on the LightCycler 480 real-time PCR platform (Roche Applied Science) using pan-bacterial 16S rRNA primers (V1-forward: GYGGCGNACGGGTGAGTAA and V1-reverse: TTACCCCACCAACTAGC primers).[16] Primers were HPLC purified (Sigma-Aldrich, Dorset, UK). PCR reactions in a final volume of 20 µL contained the following: DNA extract (250 fg-250 ng), 0.3 µM forward and reverse primers, $MgCl_2$ at a final concentration of 3 µM and 10 µL (1X) LightCycler 480 High Resolution Melting Master mix containing FastStart Taq DNA polymerase, reaction buffer and dNTP mix (Roche Diagnostics; Burgess Hill, UK). Negative controls containing PCR grade water instead of bacterial DNA extract were included. The same conditions were used for all PCR

reactions: activation step at 95°C for 10 minutes followed by 30 cycles of 95°C for 10 seconds, 62°C for 10 seconds and 72°C for 10 seconds. Reactions containing nuclease-free water were also run as negative controls (https://pim-eservices.roche.com/LifeScience/Document/7fbff181-4394-e611-6a9e-00215a9b3428).

High resolution melting analysis

HRMA was performed using the the Lightcycler 480 Gene Scanning Software. Amplified DNA was heated at 95°C for 1 minute, followed by cooling at 40°C for 1 minute. HRMA was performed over the range from 65°C to 95°C, rising at 0.02 °C/s with 25 acquisitions/°C to obtain a high resolution melting profile giving an accurate melting temperature of the amplicon. Melting curves were normalised and temperature shifted allowing difference plots to be derived that compare the melting profile given by a particular bacterial species with that of another (reference) species. In this study, *Bacteroides fragilis* was used as the reference organism for generating difference plots of vancomycin-resistant and -sensitive *Enterococcus* strains. Reactions containing purified *B fragilis* DNA (10 pg) were included in each PCR run. Analytical sensitivity of the RT-PCR-HRMA assay was determined over a million-fold range (250 fg to 250 ng) by serial dilution of DNA from vancomycin sensitive and resistant strains.

RESULTS

Confirmation of expression of vanA *or* vanB *gene*

A real-time PCR assay was optimised and used to detect the genotype of vancomycin-resistant *E faecalis* and *E faecium* strains used in this study (the presence or absence of *van* genes based on gene-specific primers for confirmation of *van* genes expression using previously published specific primer sets *vanA* and *vanB*).[17] The resistance and expression of *vanA* (*E faecium* ATCC 19434 and *E faecalis* ATCC 29212) and *vanB* (*E faecalis* ATCC 51299), the standard vancomycin-resistant strains of *Enterococcus* species used in this study, were confirmed. However, no amplification was seen with any of the standard vancomycin-sensitive strains of Enterococci (*E faecalis* NCTC 77, *E faecium* NCIMB 2699) using *vanA* and *vanB* genes primer sets (**Table 2**).

Differentiation of VRE and VSE Strains by RT-PCR-HRMA approach

Initial studies used standard strains of vancomycin-resistant and -sensitive *E faecalis* and *E faecium* to validate the RT-PCR-HRMA approach. Both vancomycin-sensitive and resistant strains used in this study were amplified with equal efficiency by the V1 16S rRNA primers. **Figure 1** shows the PCR-HRMA amplification curves of some vancomycin resistant (VRE) and sensitive (VSE) *Enterococcus* species used in this study as well as illustrates the amplification curve for *B fragilis*. However, no amplification was seen for nuclease-free water (Negative control).

Table 2. Results of *van* genotyping for VSE and VRE standard strains.

Reference strains		*van* genotype
E faecalis	NCTC 77	Negative
E faecium	NCIMB 2699	Negative
E faecium	ATCC 19434	*vanA*
E faecalis	ATCC 29212	*vanA*
E faecalis	ATCC 51299	*vanB*

Figure 2 shows results following PCR analysis by HRMA. Difference plots were generated for each organism (vancomycin-resistant and -sensitive *E faecalis* and *E faecium*) by subtracting their fluorescence melting profile from that of a reference species *B fragilis*. A characteristic difference curve shape was produced by vancomycin-sensitive strains of *E faecalis* and *E faecium*. Vancomycin-resistant strains of these bacteria were associated with a markedly different curve shape facilitating a simple differentiation of resistant and sensitive strains. These data provide evidence that amplification of a region of the 16S followed by HRMA analysis can be used to differentiate vancomycin-resistant *E faecalis* and *E faecium* with *vanA* and *vanB* genotypes from vancomycin sensitive organisms.

Figures 3 and 4 demonstrate the effect of DNA concentration on amplification curve and difference curve shape, consecutively, produced by the vancomycin-resistant *Enterococcus* strain (e.g. *E faecalis* ATCC 29212). All DNA concentrations for vancomycin-resistant *E faecalis* (ATCC 29212) ranging from 250 ng down to 250 fg were amplified using PCR-HRMA and V1 primer set (**Figure 3**). The difference curve shape was highly reproducible and no significant variation in shape was observed over a million-fold range of DNA concentration (250 fg to 250 ng per assay) (**Figure 4**). Similar data were obtained with the other reference VRE and wild-type *Enterococcus* strains used in this study (data not shown).

DISCUSSION

As described previously, Ozbak and co-authors[15] provided evidence that the PCR-HRMA approach has

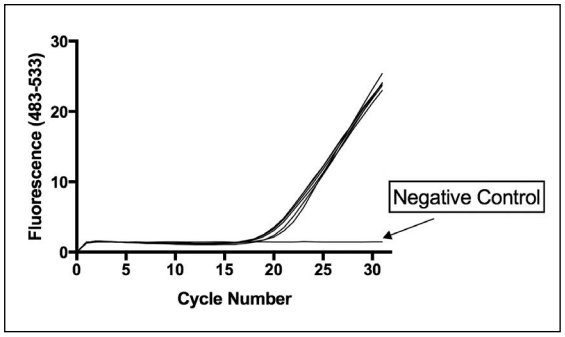

Figure 1. PCR-HRMA amplification curves of vancomycin-resistant (VRE) and -sensitive (VSE) *E faecalis* and *E faecium*. Amplification curves for VRE (*E faecium* ATCC 19434, *E faecalis* ATCC 51299 and *E faecalis* ATCC 29212) and the VSE standard strains (*E faecalis* NCTC 77 and *E faecium* NCIMB 2699). Each assay contained 250 pg of bacterial DNA. *B fragilis* was also included as the reference organism for generating VRE/VSE difference curves. *B fragilis* amplifies with the same efficiency as the Enterococcus strains. Nuclease-free water was used as a negative control (NC).

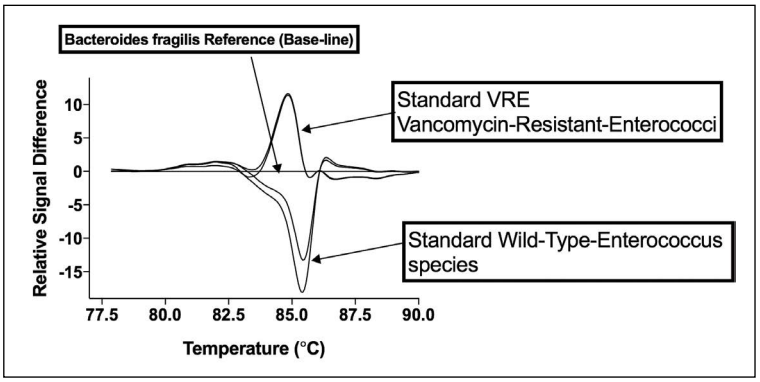

Figure 2. PCR-HRMA difference plots of VRE and VSE *E faecalis* and *E faecium* generated against *B fragilis* using V1 primer set. Difference curve plots for (A) VSE (NCTC 77, NCIMB 2699) and (B) vanA/B VRE (ATCC 19434, ATCC 51299, ATCC 29212) standard strains of *E faecalis* and *E faecium*. Each assay contained 250 pg of bacterial DNA. *B fragilis* was also included as the reference organism for generating VRE/VSE difference curves (plots). *B fragilis* amplifies with the same efficiency as the *E faecalis* and *E faecium* strains.

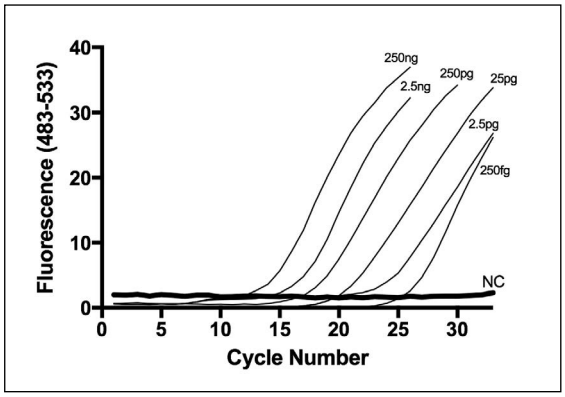

Figure 3. Concentration dependence of RT-PCR-HRMA using the V1 primer set: Amplification curves for different quantities of DNA from VRE *E faecalis* ATCC 29212 (250 fg to 250 ng) using the V1 primer pair.

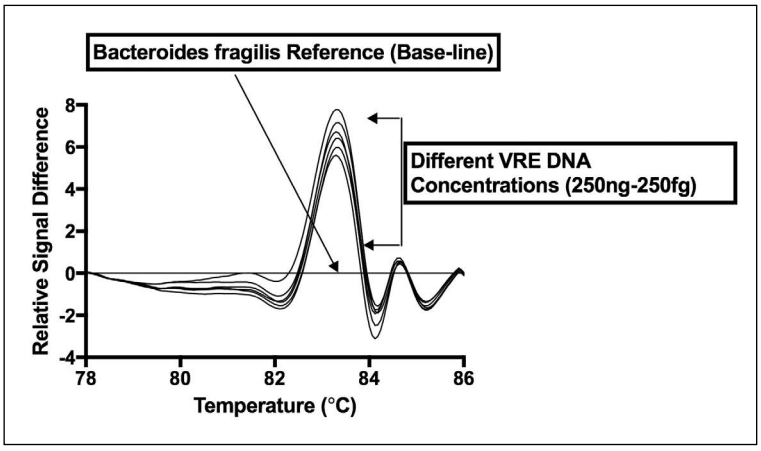

Figure 4. Analytical sensitivity of VRE PCR-HRMA assay using the V1 primer set. Data show that the characteristic difference curve shape for VRE (using DNA from VRE *E faecalis* strain ATCC 29212) is maintained over a 6-log concentration range (250 fg to 250 ng DNA).

potential as a low-cost alternative to other molecular approaches for identification of the 21 bacterial pathogens responsible for bloodstream infection (including *E faecalis* and *E faecium*). When combined with a suitable pathogen pre-amplification step for blood culture bottles, PCR-HRMA can detect and speciate low-level infection (e.g. 10 CFU/mL) and significantly faster than would be possible by the traditional clinical microbiological approach. However, a deficiency of most current molecular approaches to pathogen detection is that they often provide very limited information on antibiotic susceptibility.[17,18] Currently, antibiotic susceptibility determined by standard culture techniques is routinely provided to clinicians to help direct treatment before more specific information becomes available. As with detection and speciation of pathogens, molecular determination of antibiotic resistance has the potential to decrease significantly the time required for determination of antibiotic resistance. We studied the ability of the 16S rRNA based PCR-HRMA approach in the detection of VRE, which is one of the most important antibiotic resistances encountered in the setting of bloodstream infection. Data presented in this study suggests that the 16S PCR-HRMA assay has the potential for rapid identification of vancomycin-resistant *E faecalis* and *E faecium*. This speculation needs to be confirmed in a representative clinical sample and supported by appropriate statistical analysis.

The use of PCR-based DNA detection of antibiotic resistance is possible if the genetic differences in the genes that confer resistance are known. Molecular approaches have been established for detection of antibiotic resistant genes using mainly PCR techniques,[17,18] but also microarray.[19,20] For instance, direct detection of the *mecA* gene, the primary determinant of methicillin resistance, has been described in several studies, but most require confirmation of positive culture before detection.[21,22] These assays reliably detect *mecA* expression. The gene *mecA* is located on the chromosome but not on plasmids.[23] Furthermore, in most cases, PCR assays for MRSA are limited by an inability to differentiate MRSA from methicillin-resistant coagulase negative *Staphylococci* (CoNS) requiring other assays to differentiate *S aureus* from CoNS (usually culture) which adds to the assay time. Similar limitations apply to current PCR-based approaches to detection of ESBLs and VRE. Several assays have been described, but these require confirmation and identification of the presence of gram-negative organisms (for ESBLs) or gram-positive *Enterococcus* species (for VRE) in the sample by additional techniques prior to analysis of resistance genes.[24-26] Moreover, PCR-based approaches have been also developed for detection of *van* genes in VRE including commercial assays aimed primarily at screening of fecal samples and rectal swabs.[11,12] However, these assays lack sensitivity and have been applied only to rectal or faecal specimens. Traditional and automated antibiotic sensitivity testing methods are not able to detect low-level vancomycin resistant Enterococci (mainly *vanC* genotype) and certain strains of *vanB* genotype, which indicates the need for developing rapid molecular approaches to detect all strains of VRE in clinical samples.[27,28]

The advantages offered by the PCR-HRMA approach, including speed, relative technical simplicity compared to probe-based techniques and low cost,[13-15] makes it an attractive potential approach for detection of antibiotic resistance. HRMA has been used successfully to identify resistant genes in several bacteria. Recently, Hemarajata and his team developed in 2015 a HRMA-RT-PCR for detection and differentiation of OXA-48-Like-β-lactamases in carbapenem-resistant Enterobacteriaceae.[29] Other applications of HRMA in this area include detection of rifaximin-resistance in *Clostridium difficile*,[30] identification of nosocomial ESBL-producing *E coli* including ST131,[31] detection of antimicrobial resistance in *Neisseria gonorrhoeae*,[32] and detection of sequence type 131 *E coli*.[33] These PCR-HRMA approaches have the potential to be used as tests to determine efficiently and rapidly some antibiotic resistance genes in pathogens and therefore could facilitate the timely administration of appropriate antimicrobial therapy for the infected patient.

The decision to investigate the potential application of 16S PCR-HRMA for the detection of common antibiotic resistance mechanisms relevant to bloodstream pathogens is based on several factors. Firstly, there is emerging evidence that signals for resistance to several antibiotics can be found on the 16S ribosomal gene. Markers of antibiotic resistance can be identified from sequence analysis of this region or some resistant genes may be located on the 16S ribosomal gene.[34-36] Richardson reported that identification of antibiotic resistance is possible for some species using 16S rRNA sequential and thermodynamic properties.[34] Several plasmid-encoded 16S rRNA methylases have emerged in clinical isolates of gram-negative bacilli.[35,37] The emergence of pan-aminoglycoside-resistant, 16S rRNA methylase-producing, gram-negative bacteria has been increasingly reported in recent years.[35,37] Secondly, if signals for antibiotic resistance were found using 16S-based PCR-HRMA it would be possible to develop a combined HRMA assay that was able to identify the pathogen species (as detailed by Ozbak)[15] and at the same time provide evidence for antibiotic resistance without separate or additional steps. This is an important goal for molecular diagnostic assays of infection. For instance, SeptiFast (Roche Diagnostics), a multiplex real-time PCR platform that is designed to detect and identify a range of sepsis-related pathogens in blood, includes MRSA identification based on *mecA*[38] but this requires a second PCR assay and no other antibiotic resistances are covered. Similarly, VYOO from SIRS-Lab is designed to detect a wide range of sepsis pathogens and includes several resistance genes including *mecA* and *van* genes but there are currently no published studies of the clinical performance of this test. I was fortunate in being able to test the usefulness of HRMA to detect resistance signals because of the availability of several different 16S primer sets that had already been optimised for HRMA and shown to work well for pathogen speciation in reports by Ozbak and colleagues in 2012.[15]

The data presented in this report suggest that the 16S PCR-HRMA approach developed for pathogen speciation has the potential to reliably differentiate vancomycin-resistant *E faecalis* and *E faecium* strains carrying the most common *van* genes (*vanA* and *vanB*), from vancomycin-sensitive strains using the V1 16S primers based on differences in melting curve shapes that are generated against *B fragilis*.

The finding of a "signature" for VRE in the 16S re-

gion is interesting and unexpected. The signature does not likely represent a sequence difference due to the presence of *van* genes. These genes are associated with plasmid uptake and are not located in the 16S region, but they are located on plasmids or on the chromosome.[39,40] The implication is that HRMA is picking up a secondary change in the 16S region perhaps due to a phenotypic difference in Enterococci in association with the acquisition of *van* genes. An alternative explanation is that the gene encoding on different parts of the bacterial genome account for differences in detection, which could be due to their mobility.

Interestingly, strains with different *van* genotypes produce similar curve shapes. This appears to be a novel finding with no other studies showing a "VRE-signal" in the 16S region. Further studies are required to confirm the importance of this finding. The differences in curve shape in HRMA should reflect sequence differences in the amplified DNA. It will be important therefore to sequence the 16S product from vancomycin-sensitive and -resistant organisms including both reference standards and clinical isolates, to try to determine whether a sequence variation exists and whether the location is within the 16S gene. If a sequence difference is present in resistant organisms, further work is necessary to identify the phenotypic consequence of this variation and how it relates to vancomycin resistance. This could provide a better understanding of the secondary effects of acquiring resistance genes such as *van*. It would also be advantageous to apply this assay to a broader range of clinical isolates. Further testing of the developed VRE-HRMA assay in this study on clinical isolates of *Enterococcus* species is important for validation of this assay. It will also be advantageous to show that the same differences in melting curve shape are observed when resistant and sensitive isolates from different clinical sites are analysed using the 16S PCR-HRMA assay. Moreover, it is important to investigate whether this approach would also be useful in identifying vancomycin resistant *E casseliflavus* and *E gallinarum* expressing the *vanC* gene. It would be interesting if both *E casseliflavus* and *E gallinarum* showed the same curve shape as vancomycin-resistant *E faecium* and *E faecalis*.

In conclusion, this work has shown the potential application of the 16S PCR-HRMA approach for rapidly detecting and identifying resistant pathogens, notably vancomycin-resistant *E faecium* and *E faecalis*. The data on VRE provide a basis for combining VRE identification with pathogen speciation (as described by Ozbak[15]) in a rapid, low cost assay that is able to identify an organism as an *Enterococcus* and determine whether *E faecium* or *E faecalis* is resistant to vancomycin. This is not possible in current microbiological testing where identification of pathogens and antibiotic sensitivity require quite different and separate approaches that take a considerable time. The cost per sample in a single PCR-HRMA run (including DNA extraction, HRMA master mix and primer pairs) is estimated as about $10 (USD)[15] which is quite cheap especially when compared to other advanced molecular approaches such as sequencing. The results presented in this study suggest that 16S-PCR-HRMA assay could be useful for epidemiological studies on *Enterococcus* genotypic distribution and therefore alert us to the dissemination of resistance. This study has also raised interesting questions about whether the acquisition of *van* genes brings about secondary changes in the bacterial genome.

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