IJAMBR 2 (2014) 1-10 ISSN 2053-1818

International Journal of Applied Microbiology and Biotechnology Research

www.bluepenjournals.org/ijambr

Development and validation of a new diagnostic PCR assay for *vanA* gene encoding vancomycin resistance in *Staphylococcus aureus*

Charles Emeka Okolie[1,2]*, Alan Cockayne[1], Karl Wooldridge[1] and Richard James[1]

[1]Centre for Healthcare Associated Infections, Centre for Biomolecular Sciences Building, University of Nottingham, University Park, Nottingham, NG7 2RD, United Kingdom.
[2]Department of Biological Sciences, College of Science and Engineering, Landmark University, Omu-Aran, Kwara State, Nigeria.

Article History
Received 09 December, 2013
Received in revised form 26 December, 2013
Accepted 06 January, 2014

Key words:
Vancomycin-resistant *Staphylococcus aureus* (VRSA), *vanA*, Genetic testing, Diagnostic PCR, Methicillin-resistant *Staphylococcus aureus* (MRSA).

Article Type
Full Length Research Article

ABSTRACT

***Staphylococcus aureus* is associated with several human diseases. Following the introduction of meticillin for the treatment of infections associated with penicillin-resistant *Staphylococcus aureus*, meticillin resistant *S. aureus* (MRSA) emerged. Recent reports indicate that some MRSA strains have acquired *vanA* gene encoding staphylococcal vancomycin resistance, thus constituting further pressures on clinical antibiotic use and infection control. To prevent the spread of such clones, rapid diagnostic assays are needed. We used all the *vanA* nucleotide sequences available at the National Centre for Biotechnology Information to perform multiple alignments in the clustalW suite at the European Bioinformatics Institute from which we designed and developed a new vanA PCR assay. The new *vanA* PCR assay generated 235bp amplicon through 40-cycles in 55 min with negative predictive value of 100% and positive predictive value of 100% and sensitivity of one colony-forming unit. Following primary isolation, the turn-around time was 2.5 h against ≥ 24 h by bacteriological methods. The new assay was validated on reference vancomycin-susceptible and vancomycin-resistant staphylococcal strains (N=345) obtained from local, national and international standard supplies. PCR products were confirmed by nucleotide sequencing. Staphylococcal vancomycin resistance testing methods recently recommended by the CLSI were used to confirm staphylococcal vancomycin resistant phenotype. Use of multiple sequence data for oligo-design will help stay ahead of the bugs. The new *vanA* PCR assay can easily be integrated into the routine microbiology workflow especially for laboratories with slim budgets. Richer laboratories could easily convert the new *vanA* PCR assay from end-point platform to real-time PCR using fluorescent probes.**

INTRODUCTION

Staphylococcus aureus has long been associated with diverse diseases of humans and other animals including pneumonia, mastitis, gastroenteritis, bacteraemia, brain abscess and musculoskeletal disorders. *S. aureus* infections used to be treated successfully with penicillins, a large group of antibacterial agents which includes Penicillin G and meticillin. Before the emergence of meticillin-resistant *S. aureus* (MRSA), meticillin was found to be active against strains of staphylococci

*Corresponding author. E-mail: charlesokolie@hotmail.com, okolie.charles@lmu.edu.ng.

resistant to penicillin G (Stewart et al., 1960). Following the broad-spectrum inactivation of penicillins and related antibiotics, including meticillin, by MRSA, the glycopeptide vancomycin was introduced in the USA in 1956 (Schneierson et al., 1958) and is widely used against MRSA (Chambers, 1991; Stevens, 2006).

The first clinical strain of MRSA with reduced vancomycin susceptibility, described as vancomycin-intermediate *S. aureus* (VISA), was isolated from a surgical wound infection in Japan (Hiramatsu et al., 1997a). Subsequently, dissemination in Japanese hospitals of strains of *S. aureus* heterogeneously resistant to vancomycin, including strains with vancomycin minimum inhibitory concentration (MIC) ≥ 8 mg/L, was reported (Hanaki et al., 1998, Hiramatsu et al., 1997b). In June 2002, vancomycin-resistant *S. aureus* (VRSA) with MIC ≥ 32 µg/mL was isolated from a swab obtained from a catheter exit site from a Michigan resident aged 40 years with diabetes, peripheral vascular disease and chronic renal failure (CDC, 2002). Subsequently, several other VRSA have been reported including the VRSA isolated from a patient in Pennsylvania (Tenover et al., 2004). Aside from the VRSA isolated within the USA between 2002 and 2006 (Sievert et al., 2008), there are reports of VRSA from several parts of the world including Brazil (Lutz et al., 2003), India (Tiwari and Sen, 2006), Italy (Chesi et al., 2006) and Japan (Haraga et al., 2002).

The Centre for Disease Control (CDC) team on the Michigan diabetic reported that the DNA sequence of the *vanA* gene from the VRSA isolate was identical to the *vanA* sequence of transposon Tn*1546* and to the *vanA* sequence from the patient's *Enterococcus faecalis* isolate (Chang et al., 2003). Evolutionary genomics have shown that reservoir of MRSA and vancomycin-resistant *Enterococcus*, especially in wounds, chronic, rehabilitation and intensive care patients have been associated with the genomic escalation from MRSA to VRSA (Kobayashi et al., 2012; Zhu et al., 2010; Flannery et al., 2011; Rabinowitz et al., 2012; Thati et al., 2011). During co-habitation in such environments, MRSA acquire the *vanA* gene from *Enterococcus* species (Freitas et al., 2013; Gardete et al., 2012). Isolation of *E. faecalis* and *E. faecium* from human clinical specimens containing plasmids harbouring the entire vancomycin resistance gene cluster has been documented (Sharifi et al., 2012); while a recent report from North-eastern Iran described the phenotypic and genetic characterization of VRSA and *E. faecalis* ATCC 52199 from a patient which they demonstrated by sequencing and detection of a 713bp PCR product from the *vanA* gene (Azimian et al., 2012).

Given that vancomycin had been the mainstay of MRSA treatment in some parts of the world (Gemmell, 2004; Stevens, 2006), the emergence of vancomycin resistance in MRSA had posed a new level of threat to diagnostic microbiology, hospital infection control and antimicrobial chemotherapy (Hiramatsu, 1998; Linden, 2008). Recent expert reviews have highlighted the need for more reliable and rapid genetic testing methods (Linden, 2008; Howden et al., 2010). In response to the highlighted need, we used multiple alignments to identify highly conserved motifs (HCMs) within the *vanA* gene sequences available at the National Centre for Biotechnology Information (NCBI). The HCMs were used to design and develop a set of PCR primers for the detection of 235bp amplicon from the *vanA* gene. The new *vanA* PCR assay was validated by sequencing of PCR amplification products and by conventional phenotypic techniques.

MATERIALS AND METHODS

Bacterial isolates, culture media and culture conditions

All bacterial isolates used in this study (N=345) were sub-cultured from the strain collections used for DNA microarray work in our laboratory (Spence et al., 2008). They include 53 reference strains obtained from the Network for Antibiotic Resistance in *S. aureus* (NARSA), the National Collection of Type Cultures (NCTC) and the National Centre for Industrial and Marine Bacteria (NCIMB). The others (n=292) were sub-cultured Nottingham local clinical staphylococcal strains. Staphylococcal strains obtained from the NARSA were isolated in the Biosafety Level 3 (BSL3) suite provided for work with such potentially hazardous (multi-drug resistant and hyper-virulent) bugs. A fine touch of the frozen stock (-80°C) was inoculated into brain heart infusion (BHI) broth (Oxoid, UK). Broth cultures were shaker incubated overnight (approximately 14-24 h) at 37°C, Gram stained and sub-cultured unto BHI plates incubated (37°C, overnight) for discrete colonies.

Preparation of Inoculum turbidity standard

The British Society for Antimicrobial Chemotherapy (BSAC) standardized disk susceptibility testing method version 6 (Andrews and BSAC, 2007) and the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012) were used to prepare $BaSO_4$ turbidity standard (0.5 McFarland standard). Briefly, 0.5 mL of solution A (0.048 M $BaCl_2$ [1.17% w/v $BaCl_2$. $2H_2O$]) was added to 99.5 mL of solution B (0.18 M H_2SO_4 [1% v/v]) with constant stirring. Using matched cuvettes with a 1.0 cm light path, the $OD_{(625nm)}$ was measured on the spectrophotometer (BioMate3: ThermoSpectronic, NY, USA), blanking with water. The 0.5 McFarland standard was distributed into disposable screw-capped bijou tube

(Sterilin, UK) of the same size and volume as those to be used for matching the direct colony suspensions. The tubes were labelled with the date of preparation and the expiry date which is six months from the date of preparation, sealed tightly to prevent loss by evaporation and stored at room temperature protected from light. Before use, the inoculum standard was vigorously agitated on a vortex mixer and the absorbance was read to ensure stability.

Preparation of standardized bacterial inoculum

Using the 0.5 McFarland standard as a guide, a standardized bacterial inoculum was prepared by the direct colony suspension method (Collee et al., 1996) as approved by the BSAC and CLSI. From BHI plate, 4 discrete colonies were re-suspended in sterile distilled water to equivalence of 0.5 McFarland standard and maintained briefly on a shaker incubator at 35°C to homogenize. The bacterial density which gave an $OD_{(625nm)}$ equivalent to that of 0.5 McFarland standard is referred to as the standardised inoculum. The standardised inoculum was used within 15 min for diverse applications including antibiotic susceptibility testing (AST) and for PCR.

Design of PCR primers

The primer design strategy is detailed in an unpublished doctoral thesis (Okolie, 2009). Briefly, all published *vanA* nucleotide sequences were sourced from the NCBI and used for multiple alignments in the clustalW suite (www.ebi.ac.uk/tools/clustalW). Common subsequences were located manually as described elsewhere (Mount, 2004). Following identity search by BLAST (www.ncbi.nlm.nih.gov/BLAST/), the primers were purchased from Sigma (Sigma Genosys, Cambridge, UK) whose DNA calculator (www.sigma-genosys.com/calc/DNACalc.asp) was used to weight the oligo sequences.

Detection of bacterial DNA using the new *vanA* PCR assay

Extraction of bacterial DNA was performed by a simple heat method (Okolie, 2009). Briefly, standardized bacterial suspension (0.5 mL) was heated for 10 min at 98°C. Following centrifugation (13000 g, 20 s), the supernatant was used as template DNA for PCR amplification on an Eppendorfmastercycler (Eppendorf, Hamburg, Germany). The cycling conditions were described elsewhere (Nakagawa et al., 2005) with some modifications. Briefly, an initial single cycle for 4 min at 94°C was followed by 40 cycles consisting of 15 s at 94°C and 10 s at 60°C with a final polymerisation at 72°C for 1 min and cooling at 8°C before gel electrophoresis. PCR optimization was performed as described elsewhere (McPherson and Moller, 2006; Sahdev et al., 2007). PCR products were resolved by horizontal submarine electrophoresis (Bio-Rad, USA) at 200 V for 30 min in Agarose gel (2%) containing ethidium bromide (0.5 µg/mL), visualized using UV-transilluminator (UVP, Cambridge, UK) and the gel images were captured with the associated VisionWorks™software.

Nucleotide sequencing and genotypic identity of PCR products

PCR products were purified from Agarose gels using GenElute™ according to the manufacturer (Sigma, UK). Sequencing reactions were prepared following the Applied BiosystemsBigDye™protocol. Sequencing was performed on a Prism 310 Genetic analyser (Abbott Laboratories, USA). Identity of the sequence data obtained was analysed by BLAST.

Phenotypic bacteriological tests used to support gene detection assays

Except otherwise indicated, all media and reagents used for bacteriological work were supplied by the UK dehydrated bacteriological media supplier (Oxoid, Basingstoke, UK). Following growth on BHI plates to obtain discrete colonies, 10 mL of BHI broth was inoculated with four discrete colonies homogenously emulsified in the broth. The broth was incubated to a heavy growth (8 h) for Gram stain (identification). A standardized 0.5 MacFarland inoculum was made by diluting the heavily grown suspension and used for inoculation of a tube coagulase test and for antibiotic susceptibility tests by vancomycin agar screen (VAS) and disk diffusion (DD).

Visual analogue scale (VAS) test was performed according to CLSI recommendations (CLSI, 2012). Briefly, autoclaved BHI agar was allowed to cool down to 50°C in the water bath. Vancomycin supplement from Oxoid was incorporated to a concentration of 6.0 µg/mL. Four plates were inoculated per test sample to provide duplicate plates for the two temperature zones (30 and 35°C). To obtain an inoculum with 10^4 CFU, the standardized inoculum (10 µL) was diluted into 900µL of sterile normal saline. VAS plates were then inoculated with a 10 µL drop of the diluted inoculum by using a micropipette to deliver onto the agar surface, spotting an area of 10 to 15 mm at the centre of the VAS test plate. All plates were incubated for 24 h. As soon as they were inoculated, one set of duplicate plates were incubated at

30°C, while the second set of duplicate plates were incubated at 35°C. VAS test plates were examined carefully for evidence of small colonies (>1 colony) or a film of growth, suggesting reduced susceptibility to vancomycin.

Disk diffusion (DD) tests of oxacillin (1 µg), cefoxitin (30µg) and vancomycin (5 µg) were performed using the disk diffusion method of Kirby-Bauer according to the version 4 of the BSAC standardized disc susceptibility testing (Andrews and BSAC, 2007). Briefly, Mueller-Hinton agar plates were overlaid with the standardized inoculum of the test strain and incubated (24 h, 35°C). The inhibition zone diameters were measured and interpreted accordingly.

Determination of the sensitivity of the novel *vanA* PCR by limiting dilution method

The sensitivity (also called the limit of detection, LoD) was determined by limiting dilution experiments (dilution-to-extinction) as described in detail elsewhere (Okolie, 2009). Briefly, ten-fold serial dilutions were performed on the standardized inoculum and were used to inoculate BHI plates. For each dilution tested, 10 µL volume of bacterial inoculum was inoculated onto quadruplicate BHI plates. Colonies were counted after 24 h of incubation at 37°C and the mean of the quadruplicate was recorded. PCR tubes were inoculated with the same 10 µL volumes used to inoculate the plates. The lowest bacterial concentration whose PCR product gave a clear band on the Agarose gel was recorded as the sensitivity or LoD of the PCR assay.

Validation of the novel *vanA* PCR assay on previously characterized strains

The reliability of the new *vanA* PCR assay to detect the appropriate DNA sequence was tested by using previously characterized staphylococcal strains, 345 in number, as sources of DNA template. Reference staphylococcal isolates (n=53), including the 3 VRSA strains from NARSA, were first used to validate the novel *vanA* PCR assay. Further validation was performed on randomly selected Nottingham local clinical isolates (n=292) collected in the Nottingham area during the period from August 2003 to December 2004 and stored the laboratory (Spence et al., 2008). Validation of the new *vanA* PCR assay was conducted blindly. Without disclosing their genotypic and phenotypic properties, the new *vanA* PCR was used to test frozen stocks numerically identified as RSS001, RSS002, RSS007, etc. After testing, the results were compared against the Queen's Medical Centre (QMC) previous characterization data.

RESULTS AND DISCUSSION

Bioinformatics workup and oligo-design selects a 235bp set of *vanA* primers

Bioinformatics analysis resulted to the selection of primers for the amplification of a 235bp region from the *vanA* gene; forward sequence (vanA-F: AAGACTGCACGTTCAGGCTC) and the reverse sequence (vanA-R: GCTCGACTTCCTGATGAATACG). The 235bp marker selected for detection of the *vanA* gene following multiple alignments yielded very good results: No loss of amplification product and spurious amplification products. By using all of the *vanA* nucleotide sequences available at the NCBI at the time of design, we have offered the primers the highest chance of continuously being useful. The supplier's DNA calculator is user friendly and we believe it is highly valuable for successful oligo-design.

New *vanA* PCR assay validated by information on reference strains

The three VRSA strains previously characterized by NARSA, namely VRS1, VRS2 and VRS3, all tested *vanA*-positive by the new *vanA* PCR assay (Figure 1). Expectedly, the VRSA strains were all resistant to vancomycin by both the VAS and the DD methods. The genetic and phenotypic information available on the reference strains uphold the validity of the new *vanA* PCR in its capacity to detect the *S. aureus vanA* gene reliably (Table 1). Thus the *vanA* PCR has a positive predictive value of 100% (100% PPV). The remaining 50 reference staphylococcal strains from NARSA, NCTC and NCIMB were *vanA*-negative by the new PCR assay and susceptible to vancomycin by VAS and DD, which was 100% negative predictive value (100% NPV). The new *vanA* PCR assay did not generate false positive nor false negative results. Also, we did not encounter invalid (indeterminate) results. Invalid test results are a major cause of high turnover of approvals and withdrawals of molecular diagnostics (MDx) assays by Food and Drug Administration.

Success of the oligo-design strategy for the new *vanA* PCR assay supports the recent move towards metagenomics

One of the major challenges against the application of MDx to MRSA was that most of the assays were designed on one genome sequence. Single genome designs are ideal for gene cloning whereby only one genome needs be cloned from. In the case of MDx the outcomes of single - genome oligonucleotides were

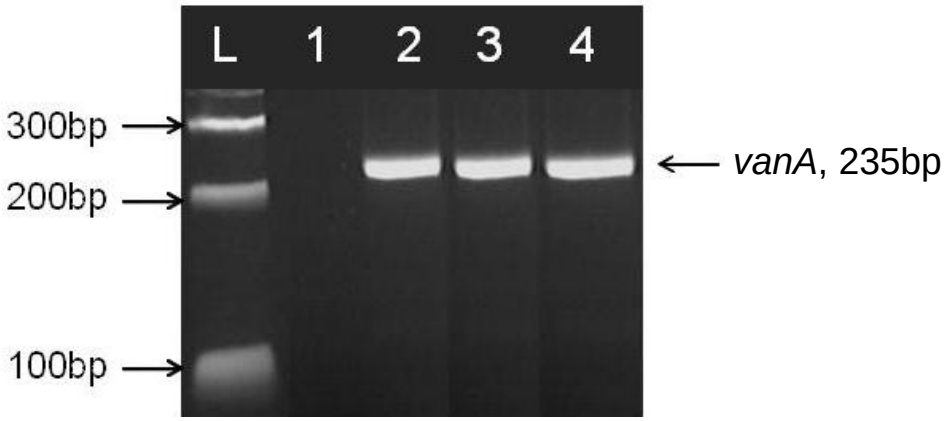

Figure 1. Specific amplification of 235bp from the *vanA* gene of VRSA. 100bp DNA Marker (Lane L), MSSA strain Sanger 476 (Lane 1), VRSA strain VRS 1 (Lane 2), VRSA strain VRS 2 (Lane 3), VRSA strain VRS 3 (Lane 4).

Table 1. Reference staphylococcal strains (n=53) from standard supplies and the results obtained on testing them using the new *vanA* PCR assay.

S/N	Isolate identity[a]	Nomenclature (species level)[b]	Source	Information at Source[c]		Experimental results[d]	
				Phenotype	*vanA* gene	Phenotype	*vanA* gene
1	NRS1	*S. aureus*	NARSA	S	_	S	_
2	NRS102	*S. aureus*	NARSA	S	_	S	_
3	NRS103	*S. aureus*	NARSA	S	_	S	_
4	NRS110	*S. aureus*	NARSA	S	_	S	_
5	NRS111	*S. aureus*	NARSA	S	_	S	_
6	NRS112	*S. aureus*	NARSA	S	_	S	_
7	NRS113	*S. aureus*	NARSA	S	_	S	_
8	NRS114	*S. aureus*	NARSA	S	_	S	_
9	NRS123	*S. aureus*	NARSA	S	_	S	_
10	NRS13	*S. aureus*	NARSA	S	_	S	_
11	NRS147	*S. aureus*	NARSA	S	_	S	_
12	NRS149	*S. aureus*	NARSA	S	_	S	_
13	NRS153	*S. aureus*	NARSA	S	_	S	_
14	NRS157	*S. aureus*	NARSA	S	_	S	_
15	NRS158	*S. aureus*	NARSA	S	_	S	_
16	NRS162	*S. aureus*	NARSA	S	_	S	_
17	NRS164	*S. aureus*	NARSA	S	_	S	_
18	NRS165	*S. aureus*	NARSA	S	_	S	_
19	NRS167	*S. aureus*	NARSA	S	_	S	_
20	NRS170	*S. aureus*	NARSA	S	_	S	_
21	NRS171	*S. aureus*	NARSA	S	_	S	_
22	NRS172	*S. aureus*	NARSA	S	_	S	_
23	NRS176	*S. aureus*	NARSA	S	_	S	_
24	NRS179	*S. aureus*	NARSA	S	_	S	_
25	NRS182	*S. aureus*	NARSA	S	_	S	_
26	NRS185	*S. aureus*	NARSA	S	_	S	_
27	NRS188	*S. aureus*	NARSA	S	_	S	_
28	NRS191	*S. aureus*	NARSA	S	_	S	_
29	NRS192	*S. aureus*	NARSA	S	_	S	_

Table 1. Continued.

S/N	Isolate Identity[a]	Nomenclature (species level)[b]	Source	Information at Source[c]		Our experimental results[d]	
				Phenotype	*vanA* gene	Phenotype	*vanA* gene
30	NRS194	*S. aureus*	NARSA	S	_	S	_
31	NRS227	*S. aureus*	NARSA	S	_	S	_
32	NRS229	*S. aureus*	NARSA	S	_	S	_
33	NRS231	*S. aureus*	NARSA	S	_	S	_
34	NRS233	*S. aureus*	NARSA	S	_	S	_
35	NRS244	*S. aureus*	NARSA	S	_	S	_
36	NRS248	*S. aureus*	NARSA	S	_	S	_
37	NRS249	*S. aureus*	NARSA	S	_	S	_
38	NRS255	*S. aureus*	NARSA	S	_	S	_
39	NRS260	*S. aureus*	NARSA	S	_	S	_
40	NRS265	*S. aureus*	NARSA	S	_	S	_
41	NRS70	*S. aureus*	NARSA	S	_	S	_
42	NRS71	*S. aureus*	NARSA	S	_	S	_
43	NRS72	*S. aureus*	NARSA	S	_	S	_
44	NRS8	*S. epidermidis*	NARSA	S	_	S	_
45	NRS9	*S. haemolyticus*	NARSA	S	_	S	_
46	NRS69	*S. haemolyticus*	NARSA	S	_	S	_
47	VRS1	*S. aureus*	NARSA	R	+	R	+
48	VRS2	*S. aureus*	NARSA	R	+	R	+
49	VRS3	*S. aureus*	NARSA	R	+	R	+
50	NCTC12217	*S. lugdunensis*	NCTC	NA	NA	S	_
51	NCTC11042	*S. haemolyticus*	NCTC	NA	NA	S	_
52	NCIMB9993	*S. epidermidis*	NCIMB	NA	NA	S	_
53	NCIMB700787	*S. capitis*	NCIMB	NA	NA	S	_

[a],Identity of isolate by type culture supplier; [b], species were identified to species level by supplier; [c], NA=information not available at source; [d], phenotype and gene detection results obtained in this study.

unfortunately very poor. Like meticillin in the history of penicillins, most of those single-genome-inspired designs were finished and being useful long before they arrived at the market. MDx assay designers need to take into consideration that a diagnostic assay designed on a given genome in one part of the world will surely need to be applied to detect sequence(s) from other genomes elsewhere. Obviously, the cost of testing large numbers of isolates is huge, but the end user wants to see an assay that generates the appropriate amplification products or signals. One cannot predict what genomes would be tested in far distant places. Therefore, the MDx assay designer would be better off using as many genomes as possible from as far and near as possible. One key contribution the metagenomics projects are likely to achieve would be that of making metagenomic data available for design and development of MDx assays. Use of multiple alignments from metagenomic data will empower more reliable sequence detection even in the face of emerging genomes. This is one of the ways to stay ahead of genome evolution. More accurate diagnostics will in turn inform more effective treatments.

New *vanA*PCR assay runs on optimized PCR factors

The optimal factors upon which the new *vanA* PCR runs currently are summarized (Table 2). The outcome of every diagnostic assay revolves around the optimization of the assay factors. Primers, dNTPs, DNA template concentration, thermo-polymerase buffer and Taq DNA polymerase were optimised to determine the optimal concentrations which generated a clearly detectable amplification product in the new *vanA* PCR assay. Optimization of PCR was a major area of research during the early days of PCR. Following its advent, the PCR process had seen several modifications and applications even in terms of the cyclers and associated materials and methodologies, including the optimization of PCR experiments.

As the PCR process extended from Cetus and Roche to several other research and development firms, a lot of improvement and optimization have been enabled. For example, the protocol for oil-overlay thermo-cyclers has been (or almost) overtaken by the heated-lid models which we used for this assay. Early researchers of PCR

Table 2. Summary of optimised PCR factors for the new *vanA* PCR assay.

Factor	Optimal value or concentration
Primers	0.75 uM
DNTPs	0.500 mM
Taq DNA polymerase	10.0 U(NEB)
Thermopolbuffer[a] (pH 8.8 at 25°C)	5 µL amounting to 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1 % Triton X-100
Time to complete a 50-cycle PCR run	90 min
Cold or Hot-start PCR	Hot-start applied

[a], Experimental laboratory maintains controlled temperature, usually ~25ºC.

optimization highlighted the influence on the fidelity of PCR by PCR factors including dNTP concentration, denaturation time and temperature, stringent annealing temperatures and magnesium chloride concentration (Tindall and Kunkel, 1988; Harris and Jones, 1997). It is no longer necessary to measure each component of the PCR thermopol buffer (Mg^{++}, Cl^-, $SO4^{--}$, Tris, etc) as they now come (already made) alongside DNA polymerases. Roche even makes it easier by supplying $MgCl_2$ along with *Taq* DNA polymerase and $MgSO_4$ with *Pwo* DNA polymerase.

Even the temperature regime has recently changed. Whereas, early PCR users were conversant with the three temperature-dependent steps of DNA denaturation, primer-template annealing and DNA synthesis by a thermostable DNA polymerase (Rychlik et al., 1990), while the new *vanA* PCR assay runs on the two temperature-driven steps recently reported for real-time PCR systems (Deurenberg et al., 2004; Nakagawa et al., 2005). Several diverse applications of PCR had been developed across all disciplines of life sciences research, and no single protocol had proven appropriate for all situations (Harris and Jones, 1997), hence the list of factors influencing PCR would never be complete (Markoulatos et al., 2002).

Speed of the new *vanA* PCR assay

Following optimization, the new *vanA* PCR completes cycling in 55 min. The total time from sample preparation to gel documentation, also called the turn-around time (TAT), was 2.5 h. This is very reasonable compared with ≥ 24 h required to identify staphylococcal vancomycin resistance following the isolation of Gram-positive cocci (GPC) in clusters. Though there is yet no commercial PCR-based assay for the detection of *vanA* gene in *S. aureus*, TAT of 2.5 h compares favourably with many commercial assays for *mecA* gene which encodes staphylococcal methicillin resistance. Diagnostic speed is especially important in blood cultures where the finding of GPC in clusters from positive blood cultures has become a source for concern to infection control epidemiologists as well as for clinical anti-staphylococcal therapy teams. We hope this speed will be of help for all the teams concerned.

Sensitivity of the new *vanA* PCR assay

The sensitivity (or LoD) of the new *vanA* PCR assay was found to be 1 CFU by limiting dilution. So long as the template DNA was applied into the PCR, even if it was only one CFU, it was amplified. Scientific literatures show a wide variation in the LoD of PCR assays. It is not uncommon to find more sensitive PCR assays than the new *vanA* PCR, especially with real-time PCR assays, in which some of which have LoD of less than 1 CFU (Nakagawa et al., 2005). Some other PCR assays are less sensitive having LoD values of ≥10 CFU (Becker et al., 1998). Sensitivity remains one of the virtues of PCR as it is able to detect very small amounts of input DNA.

Three phenotypic patterns of staphylococcal resistance to vancomycin observed

Following the performance and interpretation of the AST according to CLSI and BSAC guidelines for vancomycin by DD method, three phenotypic patterns emerged. The typical vancomycin susceptible meticillin susceptible (VSMS) phenotype is marked by clear zone of inhibition surrounding the three disks (vancomycin, oxacillin and cefoxitin) used in this study. As exemplified by *S. aureus* strain Sanger 476, all VSMS yielded no PCR amplification product when tested with the new *vanA* PCR assay (Figure 1 Lane 2). MSSAs generally fall into this group.

Another group of vancomycin susceptible staphylococci which also yielded no amplification product by the new *vanA* PCR is the vancomycin susceptible meticillin resistant (VSMR) phenotype. This group has most MRSA currently circulating in the world, and have been menacing anti-staphylococcal therapy since the advent of meticillin. *S. aureus* strains which are VSMR constitute the immediate predecessors of VRSAs (Gardete et al.,

2012).

The three VRSA strains from NARSA belong to a third group. They harbour the *vanA* gene and are phenotypically vancomycin resistant thus completely different from the two vancomycin susceptible groups. They uniformly tested positive for *vanA* by the new PCR assay (Figure 1, Lanes 2, 3 and 4) and showed the vancomycin resistant meticillin resistant (VRMR) phenotype. This group constitute a heightened threat to anti-staphylococcal therapy. Recent reviews highlighted the identification of the genetic capacity for vancomycin resistance in the staphylococci as a current need (Winstanley and Courvalin, 2011), in response to which we developed this assay.

Following on the identification of the need for rapid detection of the genetic capacity for vancomycin resistance, several laboratories have proffered DNA-based diagnostic solutions to the emerging menace of VRSA. In India, there were staphylococcal isolates from which the *mecA* gene was detected by PCR and which expressed vancomycin resistance phenotypically, but detection of *vanA* and *vanB* genes by PCR was not possible (Tiwari and Sen, 2006). However, another research group from India reported what they believed was the first *S. aureus* isolates from India having both phenotypic and genetic confirmation of vancomycin-resistance by PCR amplification of a *vanHAX*analogue (Saha et al., 2008).

In the expression of vancomycin resistance phenotype among Gram-positive bacteria, there are reports of detection of *vanAB* from enterococci (Sloan et al., 2004). However, the VRSA Investigative Team at CDC reported that PCR assays for vancomycin-resistant loci revealed only *vanA* (Chang et al., 2003; Sievert et al., 2008), thus upholding the centrality of the *vanA* gene, rather than the entire vancomycin resistance genetic island, in the mechanism of staphylococcal vancomycin resistance.

VRSA was not found in Nottingham local clinical isolates of August 2003-December 2004

VAS and DD phenotypic tests showed no expression of the vancomycin resistance phenotype among the Nottingham local strains and the 235bp marker for *vanA* gene was not detected, thus conferring a 100% negative predictive value (100% NPV) on the new *vanA* PCR assay. These findings strongly support the previous characterization data by QMC microbiology laboratory. These findings are also in agreement with the national staphylococcal vancomycin resistance data which showed that vancomycin resistance was not a problem in the UK at the time (Gemmell, 2004). The concordance between gene detection and phenotypic test results further establish the capacity of the novel *vanA* PCR assay to reliably confirm or rule out the genetic capacity for vancomycin resistance in a population of staphylococci. Our finding of concordance between PCR detection of *vanA* gene and phenotypic demonstration of staphylococcal vancomycin resistance is particularly important as recent publications have highlighted on the significance of finding Gram-positive cocci in clusters in blood cultures (Sogaard et al., 2007).

Overall performance, applicability and limitations of the new *vanA* PCR assay

The gene detection results obtained from applying the new *vanA* PCR assay corroborate the phenotypic data for the vancomycin susceptible and for the vancomycin resistant strains with 100% PPV and 100% NPV. The *vanA* primer pair detected the presence of the *vanA* gene from the genomic DNA of all the 3 (100%) *S. aureus* strains previously characterized as VRSA by NARSA. Such agreement between gene detection using the new *vanA* PCR assay and the phenotypic tests establishes the reliability of the new *vanA* PCR assay to identify *vanA*-encoded staphylococcal vancomycin resistance.

The sensitivity, speed and specificity attributes suggest that the new *vanA* PCR assay is readily adaptable for use in a routine diagnostic microbiology laboratory. With a LoD of 1 CFU, the sensitivity of the new *vanA* PCR assay makes sense in that it can be relied upon to detect as low as one CFU of *vanA* in a 40 µL PCR. The ability to speedily detect the *vanA* marker with very high specificity by application of the novel *vanA* PCR assay will allow for timely and properly guided institution of therapy and infection control measures much earlier than would be possible by application of conventional microbiological culture-based assays. Thus, our work is a positive response to the recent suggestions that DNA-based rapid diagnostic testing methods could markedly enhance therapy and infection control (Bootsma et al., 2006).

However, the new *vanA* PCR assay is not without limitation. It has been recently suggested that real-time PCR would be a more rapid diagnostic technology in the microbiology laboratory (Molenkamp et al., 2007). The cycling time of 55 min is a success. Compared with real-time PCR assays, one major limitation of the new *vanA* PCR is the time needed to resolve the PCR amplification products in Agarose gel electrophoresis. Considering the slim budget of some laboratories, especially in the developing countries, it is financially impossible at the moment for them to acquire super diagnostic machines for real-time PCR, mass spectrometry and next-generation sequencing. Therefore, this assay will find great application in the diagnostic microbiology of the developing world with tight budgets. Laboratories with fatter budgets can always increase on the speed of the new *vanA* PCR assay by use of appropriate fluorescent probe. That will exclude the gel resolution step, possibly

yielding reasonable time gains. Thus the speed limitation of the new *vanA* PCR assay is easily surmountable.

Taken together, we are happy with the performance of the new *vanA* PCR assay. We commend the NCBI, the EBI and their Japanese counterparts for their huge success in providing publicly available genomic information and tools for manipulation of genomic data.

ACKNOWLEDGEMENTS

Charles Emeka Okolie gratefully thank Dr. Harry Hoffmann Abruquah and his wife Mrs Nana Aquah Abruquah (Kwame Nkrumah University of Science and Technology) for proof-reading and making very helpful remarks on his PhD thesis and on the manuscript. All the staphylococcal strains used in this study were sub-cultured from Dr. Richard Spence's collection. For their support of his PhD, Charles Emeka Okolie is grateful to his friends and family as well as the University of Nottingham.

REFERENCES

Andrews J. M. & BSAC Working Party on Susceptibility Testing (2007). BSAC standardized disc susceptibility testing method (version 6). J Antimicrob. Chemother. 60:20-41.

Azimian A., Havaei S. A., Fazeli H., Naderi M., Ghazvini K., Samiee S. M., Soleimani M. & Peerayeh S. N. (2012). Genetic characterization of a vancomycin-resistant *S. aureus* isolate from the respiratory tract of a patient in a University Hospital in North-eastern Iran. J. Clin. Microbiol. 50(11):3581-3585.

Becker K., Roth R. & Peters G. (1998). Rapid and specific detection of toxigenic *Staphylococcus aureus*: Use of two Multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. J. Clin. Microbiol. 36:2548-2553.

Bootsma M. C., Diekmann O. &Bonten M. J. (2006). Controlling meticillin-resistant *Staphylococcus aureus*: Quantifying the effects of interventions and rapid diagnostic testing. Proc. Natl. Acad. Sci. USA. 103(14):5620-5625.

CDC (2002). *S. aureus* resistant to vancomycin: United States. MMWR. 51:565-567.

Chambers H. F. (1991).Treatment of infection and colonization caused by meticillin-resistant *Staphylococcus aureus*. Infect Control Hosp. Epidemiol. 12:29-35.

Chang S., Sievert D. M., Hageman J. C., Boulton M. L., Tenover F. C., Downes F. P., Shah S., Rudrik J. T., Pupp G. R., Brown W. J., Cardo D., Fridkin S. K. & The Vancomycin-resistant *Staphylococcus aureus* Investigative Team (2003). Infection with vancomycin-resistant *S. aureus* containing the *vanA* resistance Gene. N. Engl. J. Med. 348:1342-1347.

Chesi G., Colli A., Mestres C. A., Gambarati G., Boni F. & Gherli T. (2006). Multi-resistant-MRSA tricuspid valve infective endocarditis with ancient osteomyelitis locus. BMC Infect. Dis. 6:124.

CLSI (2012). Performance standards for antimicrobial disk susceptibility tests; Approved Standard—Eleventh Edition (M02-A11). Clinical and Laboratory Standards Institute document M2-A9, Wayne, PA, USA.

Collee J. G., Fraser A. G., Marmion B. P. & Simmons A. (1996). Mackie & McCartney Practical Medical Microbiology, New York, Churchill Livingstone.

Deurenberg R. H., Vink C., Driessen C., Bes M., London N., Etienne J. & Stobberingh E. E. (2004). Rapid detection of Panton-Valentine leukocidin from clinical isolates of *S. aureus* strains by real-time PCR. FEMS Microbiol. Lett. 240:225-228.

Flannery E. L., Wang L., Zollner S., Foxman B., Mobley H. L. T. & Mody L. (2011). Wounds, functional disability, and indwelling devices are associated with co-colonization by meticillin-resistant *S. aureus* and vancomycin-resistant Enterococci in Southeast Michigan. Clin. Infect. Dis. 53(2):1215-1222.

Freitas A. R., Novais C., Tedim A. P., Francia M. V., Baquero F., Peixe L. & Coque T. M. (2013). Microevolutionary events involving narrow host plasmids influences local fixation of vancomycin-resistance in *Enterococcus* populations. PLoS One. 8(3):e60589.

Gardete S., Kim C., Hartmann B. M., Mwangi M., Roux C. M., Dunman P. M., Chambers H. F. & Tomasz A. (2012). Genetic pathway in acquisition and loss of vancomycin resistance in MRSA strain of clonal type USA300. PLoS Pathog. 8(2):e1002505.

Gemmell C. G. (2004). Glycopeptide resistance in *Staphylococcus aureus*: Is it a real threat? J. Infect. Chemother. 10:69-75.

Hanaki H., Kuwahara-Arai K., Boyle-Vavra S., Daum R. S., Labischinski H. & Hiramatsu K. (1998). Activated cell-wall synthesis is associated with vancomycin resistance in meticillin-resistant *S. aureus* clinical strains Mu3 and Mu50. J. Antimicrob. Chemother. 42:199-209.

Haraga I., Nomura S., Fukamachi S., Ohjimi H., Hanaki H., Hiramatsu K. &Nagayama A. (2002). Emergence of vancomycin resistance during therapy against meticillin-resistant *S. aureus* in a burn patient: Importance of low-level resistance to vancomycin. Int. J. Infect. Dis. 6:302-308.

Harris S. & Jones D. B. (1997). Optimisation of the polymerase chain reaction. Br. J. Biomed. Sci. 54:166-73.

Hiramatsu K. (1998). Vancomycin resistance in staphylococci. Drug Resistance Updates. 1:135-150.

Hiramatsu K., Aritaka N., Hanaki H., Kawasaki S., Hosoda Y., Hori S., Fukuchi Y. & Kobayashi I. (1997b). Dissemination in Japanese hospitals of strains of *S. aureus* heterogeneously resistant to vancomycin.The Lancet. 350:1670-1673.

Hiramatsu K., Hanaki H., Ino T., Yabuta K., Oguri T. &Tenover F. C. (1997a). Meticillin-resistant *S. aureus* clinical strain with reduced vancomycin susceptibility.J. Antimicrob. Chemother. 40:135-136.

Howden B. P., Davies J. K., Johnson P. D. R., Stinear T. P. & Grayson M. L. (2010). Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: Resistance mechanisms, laboratory detection, and clinical implications. Clin. Microbiol. Rev. 23(1):99-139.

Kobayashi S. D., Musser. J. M. & De Leo F. R. (2012). Genomic analysis of the emergence of vancomycin-resistant *Staphylococcus aureus.* mBio. 3(4):e00170-12.

Linden P. K. (2008). Vancomycin resistance: Are there better glycopeptides coming? Expert Review of Anti-infective Therapy 6:917-928.

Lutz L., Machado A., Kuplich N. & Barth A. L. (2003). Clinical failure of vancomycin treatment of *S. aureus* infection in a tertiary care hospital in southern Brazil. Braz J. Infect. Dis. 7:224-228.

Markoulatos P., Siafakas N. & Moncany M. (2002). Multiplex polymerase chain reaction: A practical approach. J. Clin. Lab. Anal. 16:47-51.

Mc Pherson M. J. & Moller S. G. (2006). PCR. Oxford, UK. Taylor & Francis Group.

Molenkamp R., van der Ham A., Schinkel J. & Beld M. (2007). Simultaneous detection of five different DNA targets by real-time Taqman PCR using the Roche LightCycler480: Application in viral molecular diagnostics. J. Virol. Methods. 141(2):205-211.

Mount D. W. (2004). Bioinformatics: Sequence and genome analysis. New York, Cold Spring Harbor Laboratory Press.

Nakagawa S., Taneike I., Mimura D., Iwakura N., Nakayama T., Emura T., Kitatsuji M., Fujimoto A. & Yamamoto T. (2005). Gene sequences and specific detection for Panton-Valentine leukocidin.Biochem and Biophysical Research Communications. 328: 995-1002.

Okolie C. E. (2009). Development Of Diagnostic And Therapeutic Tools For *Staphylococcus aureus* Infections. PhD Thesis.University of Nottingham.Available from the British Library (http://ethos.bl.uk/OrderDetails.do?uin=uk.bl.ethos.517830) or from

the authors.
Rabinowitz R. P., Kufera J. A. & Makley M. J. (2012). A hidden reservoir of meticillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* in patients newly admitted to an acute rehabilitation hospital. PMRJ 4(1):18-22.
Rychlik W. Spencer W. J. & Rhoads R. E. (1990). Optimization of the annealing temperature for DNA amplification *in vitro*. Nucl. Acids Res. 18:6409-6412.
Saha B., Singh A. K., Ghosh A. & Bal M. (2008). Identification and characterization of a vancomycin-resistant *S. aureus* isolated from Kolkata (South Asia). J. Med. Microbiol. 57(1):72-79.
Sahdev S., Saini S., Tiwari P., Saxena S. & Singh S. K. (2007). Amplification of GC-rich genes by following a combination strategy of primer design, enhancers and modified PCR cycle conditions. Mol. Cell. Probes 21:303-307.
Schneierson S. S., Komrad E. L. & Bryer M. S. (1958).Successful treatment with vancomycin of septicemia following mitral commissurotomy caused by antibiotic resistant strain of *Staphylococcus aureus*. J. Mt. Sinai Hosp. 25:437-443.
Sharifi Y., Hasani A., Ghotaslou R., Varshochi M., Hasani A., Aghazadeh M. & Milani M. (2012). Survey of virulence determinants among vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from clinical specimens of hospitalized patients of North-west of Iran. Open Microbiol. J. 6:34-39.
Sievert D. M., Rudrik J. T., Patel J. B., McDonald L. C., Wilkins M. J. & Hageman J. C. (2008). Vancomycin-resistant *S. aureus* in the United States, 2002-2006. Clin. Infect. Dis. 46:668-674.
Sloan J. L., Uhl J. R., Vetter E. A., Schleck C. D., Harmsen W. S., Manahan J., Thompson R. L., Rosenblatt J. E. &Cockerill F. R. 3rd (2004). Comparison of the Roche Light Cycler*vanA*/*vanB* detection assay and culture for detection of vancomycin-resistant *Enterococci* from perianal swabs. J. Clin. Microbiol. 42(6):2636-2643.
Sogaard M., Norgaard M. & Schonheyder H. C. (2007). First notification of positive blood cultures and the high accuracy of the Gram stain report. J. Clin. Microbiol. 45(4):1113-1117.
Spence R. P., Wright V., Ala-Aldeen D. A. A., Turner D. P., Wooldridge K. G. & James R. (2008). Validation of virulence and epidemiology DNA microarray for identification and characterization of *S. aureus* Isolates. J. Clin. Microbiol. 46:1620-1627.
Stevens D. L. (2006). The role of vancomycin in the treatment paradigm. Clin. Infect. Dis. 42:S51-S57.
Stewart G. T., Nixon H. H., Coles H. M., Kesson C. W., Lawson D., Thomas R. G., Mishra J. N., Mitchell M. E., Semmens J. M. & Wade T. H. (1960). Report on clinical use of BRL 1241 in children with staphylococcal and streptococcal infections. Br. Med. J. 2:703-706.
Tenover F. C., Weigel L. M., Appelbaum P. C., McDougal L. K., Chaitram J., McAllister S., Clark N., Killgore G., O'hara C. M., Jevitt L., Patel J. B. &Bozdogan B. (2004). Vancomycin-resistant *S. aureus* isolate from a patient in Pennsylvania. Antimicrob. Agents Chemother. 48(1):275-280.
Thati V., Shivannavar C. T. & Gaddad S. M. (2011). Vancomycin resistance among meticillin-resistant *S. aureus* isolates from intensive care units of tertiary care hospitals in Hyderabad. Indian J. Med. Res. 134(5):704-708.
Tindall K. R. & Kunkel T. A. (1988). Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. Biochemistry 27:6008-6013.
Tiwari H. K. & Sen M. R. (2006). Emergence of vancomycin resistant *S. aureus* (VRSA) from a tertiary care hospital from northern part of India. BMC Infect. Dis. 6:156.
Winstanley T. & Courvalin P. (2011). Expert systems in clinical microbiology. Clin. Microbiol. Rev. 24(3):515-556.
Zhu W., Murray P. R., Huskins W. C., Jernigan J. A., McDonald L. C., Clark N. C., Anderson K. F., McDougal L. K., Hageman J. C., Olsen-Rasmussen M., Frace M., Alangaden G. J., Chenoweth C., Zervos M. J., Robinson-Dunn B., Schreckenberger P. C., Reller L. B., Rudrik J. T. & Patel J. B. (2010). Dissemination of an *Enterococcus* Inc18-Like *vanA* plasmid associated with vancomycin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. 54(10):4314-20.