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Development and validation of a lateral flow immunoassay for rapid detection of VanA-producing enterococci

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Background: VRE are nosocomial pathogens with an increasing incidence in recent decades. Rapid detection is crucial to reduce their spread and prevent infections and outbreaks.

Objectives: To evaluate a lateral flow immunoassay (LFIA) (called NG-Test VanA) for the rapid and reliable detection of VanA-producing VRE (VanA-VRE) from colonies and broth.

Methods: NG-Test VanA was validated on 135 well-characterized enterococcal isolates grown on Mueller–Hinton (MH) agar (including 40 VanA-VRE). Different agar plates and culture broths widely used in routine laboratories for culture of enterococci were tested.

Results: All 40 VanA-VRE clinical isolates were correctly detected in less than 15 min irrespective of the species expressing the VanA ligase and the medium used for bacterial growth. No cross-reaction was observed with any other clinically relevant ligases (VanB, C1, C2, D, E, G, L, M and N). Overall, the sensitivity and specificity of the assay were 100% for VanA-VRE grown on MH agar plates. NG-Test VanA accurately detects VanA-VRE irrespective of the culture medium (agar and broth). Band intensity was increased when using bacteria grown on vancomycin-containing culture media or on MH close to the vancomycin disc as a consequence of VanA induction. The limit of detection of the assay was 6.3×10^6 cfu per test with bacteria grown on MH plates and 4.9×10^5 cfu per test with bacteria grown on ChromID® VRE plates.

Conclusions: NG-Test VanA is efficient, rapid and easy to implement in the routine workflow of a clinical microbiology laboratory for the confirmation of VanA-VRE.

Introduction

Since their initial description in the late 1980s,[1,2] VRE have become one of the most important nosocomial pathogens worldwide, associated with increased treatment costs, prolonged hospital stay and high mortality.[3–6] The resistance can be either (i) intrinsic, as observed in *Enterococcus gallinarum* and *Enterococcus casseliflavus* that harbour a chromosomal *vanC*-like gene,[7,8] or (ii) acquired, mostly encountered in *Enterococcus faecium* and *Enterococcus faecalis*. Eight different acquired resistance genes have been described so far: *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN*.[4,6,9] In Europe, the two main genotypes are by far *vanA* and *vanB*, with VanA-producing VRE (VanA-VRE) being the most prevalent and displaying high-level resistance to both vancomycin and teicoplanin.[4,9]

The rapid detection of VRE is crucial to implement appropriate infection control measures, to adapt antibiotic treatment and to improve care strategies and outcomes.[10] Several culture-based methods, such as the use of chromogenic or selective screening media, have been developed for VRE detection from stool specimens or rectal swabs.[10,11] Detection can be optimized by inoculating clinical specimens in enrichment broth followed by sub-culture on agar plates containing vancomycin.[11,12] However, colonies grown on these selective media require confirmatory testing (such as PCR), as the specificity is low.[11,13] Molecular techniques on rectal swab specimens can shorten turnaround time,[10,14] but specificity

and positive predictive value may be low, especially for *vanB* genes that may be present in anaerobic bacteria of the intestinal microbiota, thus giving positive PCR but no culture of VanB-VRE when plating the rectal swab samples. Similarly, the recent description of *E. faecium* bearing *vanA* yet susceptible to vancomycin (vancomycin-variable *Enterococcus*), which are well detected using molecular methods, but that have no phenotypic expression and, thus, do not grow on selective media.[10,15]

Culture-based methods are long, while molecular-based ones are expensive and require specific equipment and significant expertise.[10] Lateral flow immunoassays (LFIAs) have shown their usefulness as easy, rapid and reliable confirmatory tests for detection of antibiotic resistance mechanisms, especially for β-lactamases in Gram-negatives.[16,17] An LFIA for VanA detection has already been developed, but, even though specific, it lacked sensitivity as it required sub-culturing of the bacteria on vancomycin-containing Enterococcosel agar to induce VanA expression and a time-consuming extraction protocol.[18] The aim of this study was to develop a rapid and easy to perform LFIA for the detection of VanA-VRE directly from bacteria grown on different culture media (agar and broth) widely used in routine laboratories.

Materials and methods

Ethics

All experiments were performed in compliance with French and European regulations on the care of laboratory animals (European Community Directive 86/609, French Law 2001-486, 6 June 2001) and with the agreements of the Ethics Committee of the Commissariat à l'Energie Atomique (CEtEA 'Comité d'Ethique en Expérimentation Animale' n°44) no. 12-026 and 15-055 delivered to S. Simon by the French Veterinary Services and CEA agreement D-91-272-106 from the Veterinary Inspection Department of Essonne (France).

Cloning and expression of the recombinant VanA and lysin plyV12 protein in Escherichia coli

The PCR-amplified *vanA* gene from *E. faecium* BM4147[2] using the VanA NdeI (5′-gatataCATATGaatagaataaaagttgcaatactg-3′) and the VanA XhoI (5′-gtggtgCTCGAGccccctttaacgctaatacgatcaa-3′) primers was cloned into pET22b+ vector (Novagen-Merck, Darmstadt, Germany). The synthetic purchased plyV12 gene[19] was inserted into pET22b- vector (Genecust, Boynes, France). The inserted amplicons were verified by sequencing as previously described.[13] The plasmids pET22b+ VanA and pET22b- plyV12 were transformed into *E. coli* BL 21 (DE3) pLysS and the recombinant proteins purified using Ni-NTA chelate chromatography as previously described.[17]

Monoclonal antibodies and the NG-Test VanA assay

Ten-week-old Biozzi mice were immunized by intraperitoneal injection of purified recombinant VanA protein (50 μg) as previously described.[16,17] Twenty monoclonal antibodies were tested. The best pair of antibodies were produced on a large scale and provided to NG Biotech (Guipry, France) for the development of the NG-Test VanA assay as previously described.[16,17]

Strains tested

The NG-Test VanA assay was validated using 135 well-characterized enterococci from the Associated French NRC for VRE (Hôpital Pontchaillou, Rennes, France) and from the Bicêtre Bacteriology laboratory strain collection.[13,20] This collection included 40 VanA producers, 39 VanB producers, 8 VanC1 producers, 12 VanC2 producers, 3 VanD producers, 1 VanE producer, 1 VanG producer, 1 VanL producer and 1 VanN producer and 29 non-VRE (24 WT isolates and 5 non-VRE isolates but resistant to linezolid). Ninety-four isolates were *E. faecium*, 20 isolates were *E. faecalis*, 8 isolates were *E. gallinarum* and 12 isolates were *E. casseliflavus* (Table S1, available as Supplementary data at *JAC* Online). One *E. coli* expressing VanM was used as a control. Two staphylococcal isolates (1 *Staphylococcus aureus* and 1 *Staphylococcus epidermidis*) and two carbapenemase-producing Enterobacterales (1 *E. coli* and 1 *Klebsiella pneumoniae*) were also included as negative controls.

Susceptibility testing

MICs of vancomycin, teicoplanin, linezolid and ampicillin were determined by the automated VITEK 2 system and by Etest (bioMérieux, Marcy-l'Étoile, France) and interpreted according to EUCAST guidelines.[21]

Experimental procedure

The LFIA evaluation was carried out with isolates grown overnight on Mueller–Hinton (MH) plates (Bio-Rad, Marnes-La-Coquette, France). The optimal amount of bacteria for the assay was determined by resuspending one single colony or 1×1 μL loop, 2×1 μL loops, 1×10 μL loop or 2×10 μL loops full of bacteria into 100 μL of extraction buffer (EB) provided by the manufacturer. In addition, lysozyme (10 mg/mL), lysin (80 μg/mL) and lysostaphin (12.5 μg/mL) were tested at different incubation times (5, 10 and 30 min) at room temperature and at 37°C for optimal lysis. An aliquot of 100 μL of each extract was subsequently loaded onto the cassette and the results were eye read after 15 min of migration by monitoring the appearance of a red band specific for VanA (test line, T) along with a band corresponding to the internal control (control line, C) (Figure 1).

Detection limit of NG-Test VanA

The limit of detection (LOD) was determined by using three VanA-producing vancomycin-resistant *E. faecium* isolates grown on MH or on ChromID® VRE agar plates (bioMérieux). A bacterial suspension with a turbidity equivalent to that of a 0.5 McFarland standard was serially diluted, then 100 μL of each dilution was mixed with 100 μL of EB containing 80 μg/mL lysin (EB-80) and incubated for 5 min at room temperature prior to loading onto the cassette. Serial dilutions were also plated on MH plates to determine the exact cfu/mL.

Growth culture media

The impact of the culture medium on assay performance was evaluated with nine enterococci (four VanA-VRE, three non-VanA-VRE and two non-VRE) cultured overnight on seven agar plates [MH and UriSelect™ 4 (Bio-Rad), ChromID® VRE, Columbia agar + 5% horse blood, chocolate agar PolyViteX, D-Coccosel agar (bioMérieux) and in-house prepared MH plates supplemented with 6 mg/L vancomycin (MH-vanco-6)] and in two broth media [brain heart infusion and VRE broth (bioMérieux)] widely used in routine laboratories for culturing enterococci (Table S2). For the broth, 500 μL of culture was centrifuged for 5 min at 10 000 rpm and the pellet was resuspended in 100 μL of EB-80 and incubated for 5 min at room temperature.

Results

Optimization of the experimental protocol

Five amounts of bacteria grown on MH plates (single colony or 1×1 μL loop, 2×1 μL loops, 1×10 μL loop or 2×10 μL loops full of bacteria) were resuspended in 100 μL of lysis buffer and loaded directly onto the cassettes. As shown in Figure 1(a) 1–3, a 1 μL loop full of bacteria was sufficient to obtain a weak positive signal.

Figure 1. NG-Test VanA results. (a) Obtained for *E. faecium* VanA BM4147[2] in different testing conditions: (1) 1×1 μL loop full, (2) 2×1 μL loops full and (3) 1×10 μL loop full of bacteria grown on MH, resuspended in 100 μL of EB and directly loaded onto the cassette; (4) 1×1 μL loop full of bacteria grown on MH, resuspended in 100 μL of EB and incubated for 5 min at room temperature and (5) 1×1 μL loop full of bacteria grown on MH, resuspended in 100 μL of EB-80 and incubated for 5 min at room temperature prior to loading on the cassette; and three colonies of bacteria grown on MH (6) and grown on ChromID® VRE (7) were resuspended in 100 μL of EB-80 and incubated for 5 min at room temperature prior to loading on the cassette. (b) Obtained with a 1 μL loop full of bacteria grown on MH, resuspended in 100 μL of EB and incubated for 5 min at room temperature prior to loading on the cassette. The tested bacteria were: (1) *E. faecium* VanA BM4147;[2] (2) *E. faecalis* ATCC 29212; (3) *E. faecalis* VAN B V583, (4) *E. gallinarum* VanC1 BM4174; (5) *E. casseliflavus* VanC2 ATCC 25788; (6) *E. faecium* VanA isolate 12 (vancomycin/teicoplanin MIC = 256/48 mg/L);[13] and (7) *E. faecium* VanA isolate 2 (vancomycin/teicoplanin MIC = 16/6 mg/L).[13] (c) Obtained with *E. faecium* VanA BM4147:[2] (1) 10^6 cfu, (2) 10^5 cfu and (3) 10^4 cfu from ChromID® VRE (bioMérieux); and (4) 10^7 cfu, (5) 10^6 cfu and (6) 10^5 cfu from MH. (d) Obtained with 500 μL of overnight-grown *E. faecium* VanA BM4147[2] in brain heart infusion broth (1) and in VRE broth (2) (bioMérieux) and *E. faecalis* VAN B V583[13] in brain heart infusion broth (3) and in VRE broth (4) (bioMérieux). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Increasing the amounts of bacteria increased the intensity of the band, but interfered with migration, leading to non-valid tests as the control line was not present (Figure 1a 3). Lysin, lysozyme or lysostaphin was added to the EB and incubated for 5, 10 and 30 min at room temperature and at 37°C to improve VanA extraction. The best results were obtained with an EB-80 with a 5 min incubation time at room temperature (22°C) (Figure 1a 4 and 5 and Figure 2a) prior to loading of 100 μL of this extract onto the cassette.

For bacteria grown on selective media (such as ChromID® VRE or MH-vanco-6) or on MH close to a vancomycin disc, two to three colonies were sufficient to provide a strong positive VanA signal

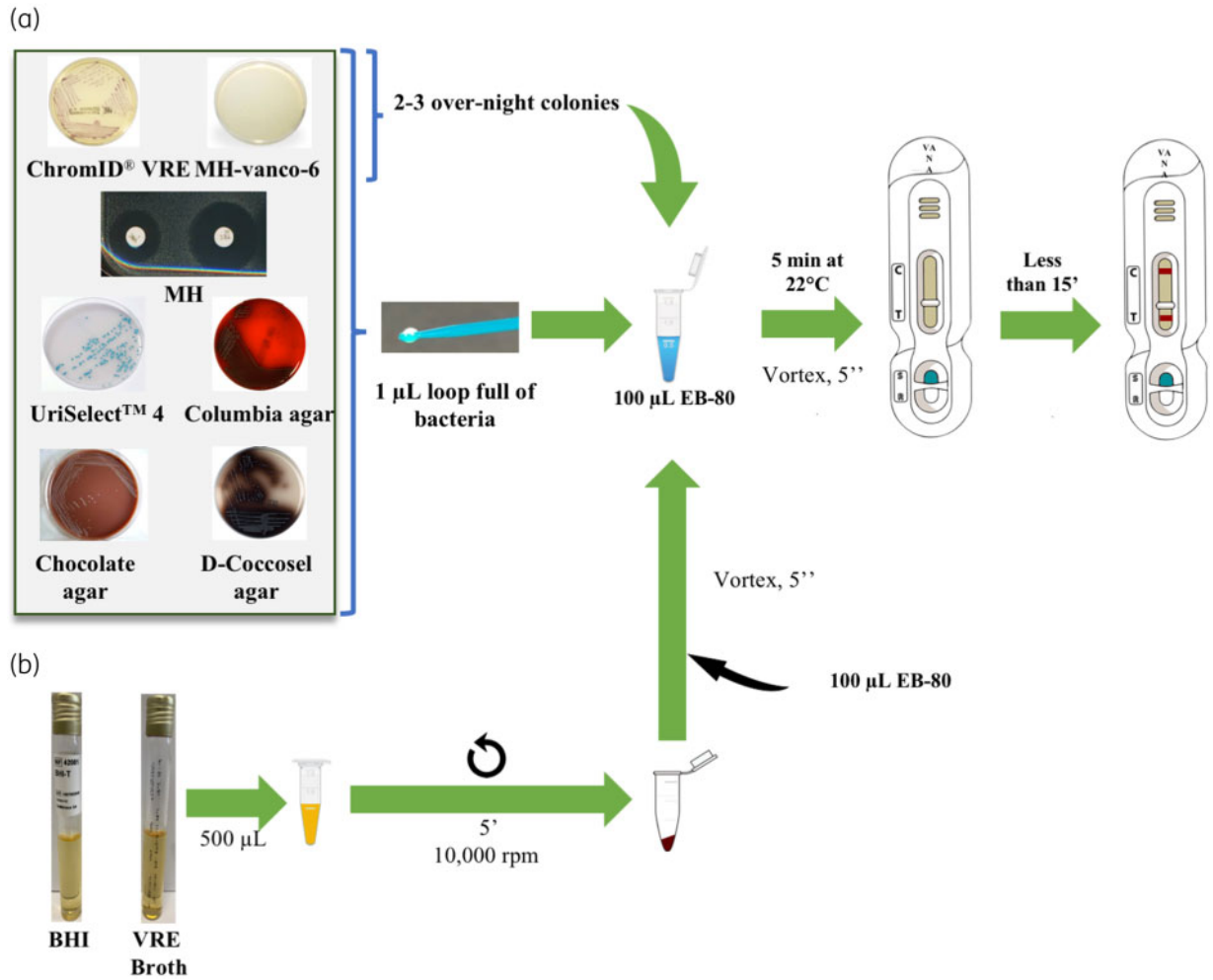

Figure 2. Experimental procedure. (a) From overnight-grown bacteria on agar plates. MH and UriSelect™ 4 were from Bio-Rad. Columbia agar + 5% horse blood, chocolate agar PolyViteX, D-Coccosel agar and ChromID® VRE were from bioMérieux. (b) From liquid broth. Brain heart infusion and VRE broth were from bioMérieux. The black cirular arrow stands for centrifugation. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

(Figure 1a 6 and 7). The use of a 1 µL loop full of bacteria did not improve the intensity, suggesting that, with two to three colonies, the detection was already optimal.

Performance of NG-Test VanA

NG-Test VanA evaluation was done with a 1 µL loop full of bacteria grown on MH plates. It was able to detect all 40 VanA-VRE with a high intensity signal (Table S1 and Figure 1b) except for 2 VanA-producing vancomycin-resistant *E. faecium* isolates that reproducibly yielded lower intensity bands (Figure 1b 7). These isolates were described as VanA genotype-VanD phenotype and display lower MICs of vancomycin (16 and 24 mg/L).[13] A high intensity signal was obtained when these two isolates were grown on ChromID® VRE (bioMérieux). No cross-reaction was observed with the other clinically relevant ligases (VanB, C, D, E, G, L, M and N) or with other Gram-positive cocci or with Enterobacterales (Table S1). Thus, NG-Test VanA displayed 100% sensitivity and 100% specificity for VanA-VRE detection.

Detection limit

The LOD was 6.3×10^6 cfu and 4.9×10^5 cfu per test with bacteria previously grown on MH and ChromID® VRE plates (containing glycopeptide), respectively (Figure 1c).

Effects of growth medium on LFIA results

Seven VRE and two non-VRE were grown on seven agar-based media widely used in routine laboratories for culturing enterococci (Table S2). NG-Test VanA was able to detect all VanA-VRE grown on non-selective media with similar band intensities, while growth on selective media (MH-vanco-6 or ChromID® VRE) increased the intensity of the signal. In addition, the staining of the colonies on chromogenic media (UriSelect™ 4 or ChromID® VRE) didn't alter the aspect of the nitrocellulose membrane or the intensity of the bands, thus allowing easy interpretation of the results. Finally, growth in liquid media such as brain heart infusion or enrichment broth (VRE broth, bioMérieux) allowed clear VanA-VRE detection (Figure 1d 1–4 and Table S2).

Discussion

Accurate and rapid detection of VRE remains challenging and yet mandatory for infection control and for treatment of infections caused by these bacteria. VanA is the most prevalent vancomycin-resistance determinant worldwide. Screening of VanA-VRE carriers may be done by spreading rectal swabs on selective culture plates. In parallel, direct screening may also be done using molecular tools, which is faster but does not replace bacterial culture.[10,17,20,22] As selective culture media have low specificity, growing colonies need to be tested for the presence of *vanA* gene using generally molecular techniques.[10,13,22]

Here, we have developed a highly sensitive and specific LFIA for VanA-VRE detection, with an easy and rapid extraction protocol suitable for routine use, that could complete/replace the molecular confirmatory tests. The LOD of NG-Test VanA was 6.3×10^6 cfu and 4.9×10^5 cfu per test with bacteria previously grown on MH and ChromID® VRE plates, respectively. The presence of vancomycin in the media, known to induce VanA ligase production through activation of the sensor VanS,[15] was able to improve the LOD by 10-fold. The NG-Test VanA assay is at least 12-fold more sensitive than the assay developed by Ji *et al.*[18] that reported an LOD of 6×10^6 cfu per test on MH-vanco-6 plates.

NG-Test VanA may be used with several culture media used to grow enterococci in clinical microbiology laboratories, as similar band intensities were observed, except for vancomycin-containing plates (ChromID® VRE and MH-vanco-6), which gave a significantly stronger band intensity. Detection of VRE from rectal samples, especially those with low carriage ($<10^4$ cfu), can be optimized by inoculating clinical specimens overnight in selective enrichment broth and subsequent sub-culturing on agar plates containing vancomycin.[11,12] As NG-Test VanA may be used directly on VRE enrichment broth, the additional 24 h sub-culturing step may be skipped. Finally, being able to work directly on bacteria grown in broth may be the premise for its use on positive blood cultures, as already shown for other LFIAs such as NG-Test Carba5, and on urinary tract infections.[23]

NG-Test VanA is easy to use, rapid, does not require any specific equipment or skills and results are easy to read after 15 min of migration. It can be easily implemented in the routine workflow of most clinical laboratories as a confirmatory test of VanA-VRE, especially from colonies grown on selective media and from enrichment broth (rectal screenings) or from antibiograms (infections) with reduced susceptibility to glycopeptides. The rapid detection (less than 15 min) will result in more efficient management of carriers and infected patients. Further studies are now necessary to evaluate NG-Test VanA on clinical samples (blood cultures and urinary tract infections) and develop tests able to detect VanB and VanM determinants, in order to include the most prevalent glycopeptide resistance mechanisms encountered in VRE.[4,10,24]

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Transparency declarations

M.L. is an NG Biotech employee mainly involved in assay development. All other authors: none to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online.

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