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Comparison of VITEK® 2, three different gradient strip tests and broth microdilution for detecting *vanB*-positive *Enterococcus faecium* isolates with low vancomycin MICs

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Objectives: In 2018, EUCAST issued a warning regarding unreliable results of gradient strip tests for confirming vancomycin resistance in enterococci. We compared the performance of various diagnostic standard and confirmatory tests to identify and determine *vanB*-type vancomycin resistance.

Methods: We analysed a collection of *vanB*-positive *Enterococcus faecium* isolates (*n*=68) with low vancomycin MICs and compared the performance of VITEK® 2 (bioMérieux), broth microdilution and three gradient strip tests from different providers (Oxoid, Liofilchem and bioMérieux). For the latter we compared the standard procedure with a protocol with increased inoculum, a rich agar medium and a longer incubation time ('macromethod').

Results: The sensitivity of VITEK® 2 was 81% compared with 72% for broth microdilution and 61%–63% for the three gradient strip tests using standard conditions. The macromethod substantially improved the performance of all strip tests resulting in a sensitivity of 89%–96% after 48 h of incubation.

Conclusions: We recommend that EUCAST changes the present warning against the general use of MIC strips. When MIC strips are used to either exclude or confirm suspected vancomycin resistance in *E. faecium*, and a PCR is not available, the macromethod should be employed. For clinically relevant enterococci, where a rapid therapeutic decision is needed, a molecular test (e.g. PCR) should be favoured in order to save time and to further increase sensitivity.

Introduction

Many hospitals in Germany and other European countries are challenged by an increasing number of VRE associated with colonizations and infections in hospitalized patients. The rising numbers of VRE are mainly driven by an increase in *vanB*-positive VRE, locally[1] or on a nationwide scale.[2] Furthermore, there is growing recognition of *vanB*-VRE with low vancomycin MICs just below the breakpoint of 4 mg/L.[3,4] Uncertain diagnostic results may rely on confirmation by alternative tests such as MIC gradient strip assays (e.g. Etest). EUCAST issued a warning in July 2018 regarding less-reliable strip assay results for determining and confirming vancomycin resistance in enterococci (http://www.eucast.org/ast_of_bacteria/warnings/), leaving the diagnostic community with uncertainty regarding how to confirm *vanB*-positive VRE with low-level vancomycin resistance. For the present study, we established a strain collection of pre-characterized and heterogeneous *vanB*-positive *Enterococcus faecium* isolates from all over Germany (*n*=68) and from recent years, which all had low vancomycin MICs in previous standard diagnostic assays and/or ambiguous resistance phenotypes. We aimed to compare the performance of five diagnostic standard and confirmatory tests to identify and determine *vanB*-type vancomycin resistance in the most reliable manner.

Materials and methods

The 68 *vanB*-positive *E. faecium* isolates originated from all over Germany and displayed low vancomycin MICs in previous diagnostic assays (see Table S1, available as Supplementary data at *JAC* Online). The collection

Table 1. Performance of different primary and confirmatory diagnostic assays in detecting *vanB*-mediated vancomycin resistance in *E. faecium* isolates (*n*=68)

Susceptibility test method	Test version	No. of VRE detected	Sensitivity (%)	Sensitivity 95% CI (%)
NA	*vanB*-PCR (reference)	68	100	95–100
Broth microdilution	24 h	41	72	61–80
	48 h	68	100	95–100
bioMérieux VITEK® 2	20 h	52	81	71–89
bioMérieux Etest®	standard (24 h)	26	62	52–71
	standard (48 h)	38	69	59–78
	macromethod (24 h)	57	86	76–93
	macromethod (48 h)	63	93	85–98
Liofilchem MIC test strip®	standard (24 h)	24	61	51–70
	standard (48 h)	32	65	55–74
	macromethod (24 h)	45	75	65–83
	macromethod (48 h)	60	89	80–95
Oxoid M.I.C. Evaluator®	standard (24 h)	28	63	53–72
	standard (48 h)	36	68	58–77
	macromethod (24 h)	58	87	77–94
	macromethod (48 h)	65	96	88–99

NA, not applicable.

Ten vancomycin-susceptible *E. faecium* (negative for *vanA* and *vanB*) served as a control group.

Specificity in all assays was 100% (95% CI 69%–100%). See the Materials and methods section for the specification of the gradient strip assay 'macromethod'. The Oxoid M.I.C. Evaluator™ provides concentrations that correspond to doubling dilutions (on a $\log_2$ basis, as for broth microdilution), whereas the Etest® and the MIC strip test® also provide 'half-dilution step' concentrations (e.g. 1.5, 3 or 6 mg/L; see also Figure 1, Table S1, Figure S1 and Figure S2). For better comparability of data, these latter concentrations were extrapolated to the next higher concentration (e.g. 3 to 4, or 6 to 8 mg/L).

was especially enriched with isolates demonstrating vancomycin MICs of 2–4 mg/L (susceptible, S) and 8 mg/L (resistant, R) in broth microdilution. We did not include *vanB* strains with vancomycin MICs of ≤1 mg/L as these strains may possess defects in *vanB* regulation.[5] To exclude any bias in the strain collection, we included isolates from 42 diagnostic laboratories, submitted between 2015 and 2018 to the National Reference Centre. We determined vancomycin MICs using the VITEK® 2 card AST P611 (bioMérieux, Nurtingen, Germany), broth microdilution (according to EUCAST standards) and gradient strip assays from three providers (M.I.C. Evaluator™, Oxoid/Thermo Fisher Scientific, Wesel, Germany; MIC test strip®, Liofilchem, Roseto degli Abruzzi, Italy; and Etest®, bioMérieux). For broth microdilution we used CAMHB from Becton-Dickinson (Heidelberg, Germany). Vancomycin (hydrochloride) powder was purchased from Sigma–Aldrich/Merck (Taufkirchen, Germany). For gradient strip assays, we compared the standard procedure versus the 'macromethod', which includes a rich agar medium (brain heart infusion instead of Mueller–Hinton; all agar media from Oxoid, Wesel, Germany), a higher inoculum (McFarland 2 instead of 0.5) and a longer incubation time of up to 48 h. *Enterococcus faecalis* ATCC 29212 (vancomycin MIC range: 1–4 mg/L), *E. faecium* ATCC 19434 (vancomycin MIC range: 0.5–2 mg/L) and *Enterococcus gallinarum* BM4174 (*vanC1*; vancomycin MIC: 8–16 mg/L) were used as reference isolates. Ten vancomycin-susceptible *E. faecium* (negative for *vanA* and *vanB*) served as a control group. Presence of *vanB* in the study group isolates and absence in control and reference isolates was confirmed by PCR.[4] Statistical calculations for sensitivity and specificity were carried out using MedCalc (https://www.medcalc.org/calc/diagnostic_test.php).

Results

The 10 vancomycin-susceptible *E. faecium* of the control group and the susceptible reference isolates were all negative for *vanA* and *vanB* and revealed MICs ≤4 mg/L in all assays and under standard conditions.

VITEK® 2 identified 52 of the 68 *vanB*-VRE (sensitivity 81%, 95% CI 71%–89%; Table 1). Using broth microdilution, only 41 *vanB*-VRE were identified after 24 h, demonstrating a sensitivity of 72% (95% CI 61%–80%) for the 'gold standard'. Incubation of the plates for another 24 h allowed the identification of all 68 *vanB*-VRE (MICs of >4 mg/L).

The comparison of three gradient strip assays revealed similar results for the vancomycin Etest, vancomycin MIC test strip and vancomycin M.I.C. Evaluator: altogether, 26, 24 and 28 *vanB*-VRE, respectively, were correctly identified leading to sensitivities of 61%–63% (Table 1 and Figure 1; see also Figures S1 and S2). Sensitivities were slightly higher after the 48 h readout (65%–69%; Table 1).

Using the macromethod (see the Materials and methods section) substantially improved the sensitivity of all gradient strip assays. After 24 h of incubation, 57 *vanB*-VRE were identified by Etest (sensitivity 86%, 95% CI 76%–93%), 45 by MIC test strip (sensitivity 75%, 95% CI 65%–83%) and 58 by M.I.C. Evaluator (sensitivity 87%, 95% CI 77%–94%). Sensitivity further improved after the 48 h readout with 63 *vanB*-VRE (93%, 95% CI 85%–98%) for Etest, 60 (89%, 95% CI 80%–95%) for MIC test strip and 65 (96%, 95% CI 88%–99%) for M.I.C. Evaluator.

Discussion

Since their first description in the early 1990s, low vancomycin MICs have predominantly been reported for *vanB*-type rather than *vanA*-type enterococci. The phenomenon is most probably caused
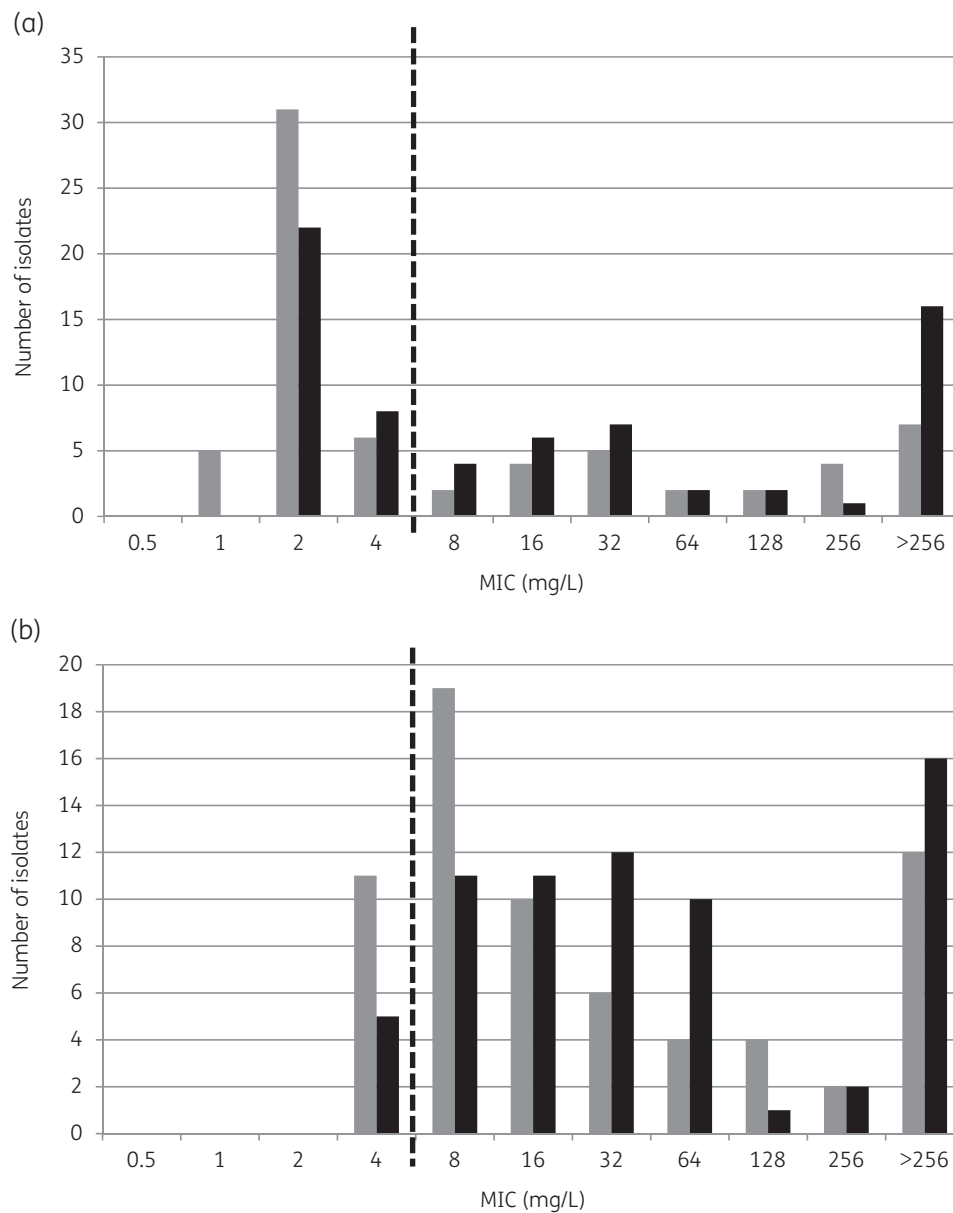
Figure 1. Performance of the Etest® (bioMérieux) by the standard method (a) versus the 'macromethod' (b) for detection of *vanB*-positive *E. faecium* (*n*=68). The broken line corresponds to the EUCAST clinical breakpoint for vancomycin (R >4 mg/L). Grey columns represent MIC results after 24 h and black columns MIC results after 48 h.

by different two-component circuits mediated via VanR/VanS and VanR$_B$/VanS$_B$ regulating induction of vancomycin resistance in *vanA*-type and *vanB*-type VRE, respectively. CLSI and EUCAST recognized this by broadening the intermediate category for vancomycin (CLSI) or lowering the vancomycin clinical breakpoint (EUCAST). However, correct identification, especially of *vanB*-type VRE, remains challenging.[4–6] The sensitivity for detection of *vanB*-type VRE was 81% for VITEK® 2 compared with 72% for broth microdilution and 61%–63% for the three gradient strip tests. The macromethod substantially improved sensitivities of all strip tests to 89%–96% after the 48 h readout. Specificity remained excellent when using the macromethod (100%, 95% CI 69%–100%).

Previous attempts to improve detection of *vanB*-type VRE included supplementation of agar media with 10 g/L oxgall (Mueller–Hinton, brain heart infusion). This increases *vanB* cluster gene expression and thereby allows improved detection of *vanB*-VRE with low vancomycin MICs.[3] Unfortunately, media with this supplement are not commercially available. Applying the macromethod with commercial kits and media might be implemented much more easily and would simply require an extended incubation of up to 48 h. We are well aware that an additional working day is in conflict with demands for quick and reliable diagnostics and for decisions for infection control, for instance, in cases of admission sample screening. Nevertheless, using the

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macromethod with 24 h of incubation was already superior to the gold standard broth microdilution. PCR-based screening directly from clinical samples may have certain advantages in identifying *vanB*-type resistance, but the reservoir and frequent occurrence of *vanB* in human intestinal commensal bacteria conflicts with a reliable test result and inevitably demands confirmation by culture.[7,8]

For VRE diagnostics, EUCAST recommends using vancomycin disc diffusion (when a PCR is not used), measuring the zone (suspect resistant if <12 mm) and noting whether the zone edge is sharp or fuzzy (suspect resistant if fuzzy) and whether there are colonies inside the inhibition zone (suspect resistant if colonies are in the zone). Either of these phenomena indicates glycopeptide resistance and a PCR should be performed to confirm or exclude the presence of *vanA* or *vanB*. A Scandinavian multicentre study revealed that vancomycin disc diffusion might be superior to other diagnostic assays in detecting low-level vancomycin resistance in *vanB*-VRE; however, a reliable execution of the tests and proper reading of the results required experienced and trained personnel.[9]

In EUCAST expert rules v3.2, which are currently (April 2019) in a wide consultation process, it is recommended that enterococci with a positive *vanB* result that appear vancomycin susceptible should be reported as being resistant to vancomycin (http://www.eucast.org/documents/consultations/). We fully support this deduction, which is in line with our study results demonstrating that the sensitivity of currently available methods needs improvement and minor methodological changes may influence the categorization of susceptible or resistant to vancomycin.

In conclusion, we recommend that EUCAST changes the present warning against the general use of MIC strips. When MIC strips are used to either exclude or confirm suspected vancomycin resistance in *E. faecium*, and a PCR is not used, the macromethod should be employed. All manufacturers of MIC strips should adapt their guidelines to this effect and should distribute this knowledge and its application properly. For clinically relevant enterococci, where a rapid therapeutic decision is needed, e.g. for isolates from bloodstream infections, a molecular test (e.g. PCR) should be favoured in order to save time and to further increase sensitivity.

Transparency declarations

None to declare.

Supplementary data

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

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