Journal of Clinical Microbiology

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J. Clin. Microbiol. **2012, 50(5):1742. DOI: 10.1128/JCM.06307-11.**
Published Ahead of Print 7 March 2012.

JCM
Journals.ASM.org

A Novel δ-Hemolysis Screening Method for Detecting Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus* and Vancomycin-Intermediate *S. aureus*

V. Cafiso, T. Bertuccio, D. Spina, S. Purrello, G. Blandino, and Stefania Stefani

Department of Bio-Medical Sciences, Microbiology, University of Catania, Catania, Italy

We assessed a new screening method, based on δ-hemolysin production in the presence of 6 mg/liter vancomycin, to distinguish heteroresistant vancomycin-intermediate *Staphylococcus aureus* (hVISA) and vancomycin-intermediate *S. aureus* (VISA) from vancomycin-susceptible *S. aureus* (VSSA). On 37 clinical methicillin-resistant *S. aureus* (MRSA) isolates, hVISA and VISA displayed no δ-hemolysis whereas VSSA displayed strong δ-hemolysis, showing 91.6% sensitivity. These data, supported by real-time reverse transcription PCR (real-time RT-PCR) highlighting an *hld* downregulation, i.e., VSSA>hVISA>VISA, define this new assay as a valid screening method.

Heteroresistant vancomycin-intermediate *Staphylococcus aureus* (hVISA) and vancomycin-intermediate *S. aureus* (VISA) represent a serious challenge in health care due to the difficulties in the treatment of their infections.

Nowadays, different screening methods are in use to detect these strains, but their identification remains difficult, controversial, and time-consuming, thus complicating their already difficult therapeutic treatment.

Our aim was to develop a simple and rapid screening method for heteroresistant vancomycin-intermediate *Staphylococcus aureus* (hVISA) and vancomycin-intermediate *Staphylococcus aureus* (VISA), based on δ-hemolysin production, to correctly identify and distinguish hVISA and VISA from vancomycin-susceptible *S. aureus* (VSSA), based on the observations that hVISA and VISA are characterized by a decreased functionality of the regulatory *agr* (accessory gene regulator) locus encoding δ-hemolysin (1, 2, 7, 8, 11–15).

We tested the ability of 37 clinical methicillin-resistant *S. aureus* (MRSA) isolates previously categorized as VSSA, hVISA, or VISA by Macro Etest and population analysis profile (PAP) analysis (4), in comparison with those obtained with the four prototypes, i.e., NRS149 (VSSA), Mu3 (hVISA), Mu50 (VISA), and NRS155 (*agr* knockout), to produce δ-hemolysin on different 5% sheep blood agar plates and to check the stability of the test on different media.

We included 24 epidemiological and genetically unrelated clinical hVISA isolates selected from 2000 to 2010 and belonging to diverse clonal groups (based on sequence type [ST], SCC*mec*, pulsed-field gel electrophoresis [PFGE], and *agr* group) on the basis of previously published data (4, 5), 5 NARSA (Network on Antimicrobial Resistance in *Staphylococcus aureus*) VISA isolates of ST5/SCC*mec*II/*agrII* according to the NARSA data (http://www.narsa.net), belonging to the same clonal group due to its natural predominance in the VISA strain population, 8 unrelated clinical VSSA strains selected based on previously published data (6), and 4 reference NARSA strains: NRS149 (VSSA *agrII*), Mu3 (hVISA *agrII*), and Mu50 (VISA *agrII*) as prototypes and controls for the phenotypic and molecular assays and NRS155 as a negative control of the *agr* functionality.

The ability to produce δ-hemolysin was measured by cross-streaking perpendicularly to *S. aureus* RN4220, which produces only β-hemolysin on sheep blood agar. δ-Hemolysin produced by a test strain resulted in a zone of enhanced hemolysis in areas where this lysis overlapped the β-hemolysin zone of RN4220 (14).

This assay was performed on different media, i.e., 5% sheep blood agar with Columbia base (COL), Mueller-Hinton base (MH), or Trypticase soy agar base (TSA) manufactured by Oxoid (OX) (Oxoid, Cambridge, United Kingdom), bioMérieux (BM) (bioMérieux, Marcy l'Étoile, France), or Becton Dickinson (BD), or homemade (HM) 5% sheep blood agar COL with 6 mg/liter vancomycin (VAN). Results were compared with those from the same formulations industrially manufactured by Liofilchem (LC) (Aquila, Italy), and were read after 24 h of incubation at 37°C. Assays were performed in triplicate. With regard to the interpretation of the tests, a semiquantification of δ-hemolysin production was performed on a scale of 0 to 3 relative to NRS149; a score of 0 (−) indicated the absence of a δ-hemolysis zone, as in NRS155, while a score of 1 (+) indicated minimal δ-hemolysin production and a score of 2 (++) or 3 (+++) indicated high or considerable δ-hemolysin activity, respectively.

Regardless of the medium-manufacturing company (OX, BM, BD), δ-hemolysin production assays performed on prototype microorganisms (Table 1) showed that, in all media used, NRS149 (VSSA) presented a large zone of δ-hemolysin, ranging from + to +++ depending on the medium used. Mu3 (hVISA) showed a smaller δ-hemolysis zone in COL and TSA blood agar (−/+), a large one in MH (++), and no δ-hemolysis zone in HM and LC blood agar with 6 mg/liter VAN; neither Mu50 (VISA) nor NRS155 exhibited a δ-hemolysis zone (−) in any of the media tested (Table 1; Fig. 1).

Therefore, the addition of 6 mg/liter of vancomycin to the COL

Received 7 November 2011 **Returned for modification** 9 November 2011
Accepted 28 February 2012
Published ahead of print 7 March 2012
Address correspondence to Stefania Stefani, stefanis@unict.it.

doi:10.1128/JCM.06307-11

TABLE 1 Results of δ-hemolysis screening

Sample or parameter	Result[a] on medium[b]								
	OX COL	BM COL	BD COL	OX MH	BM MH	OX TSA	BM TSA	BD TSA	HM and LC COL VAN
Prototype strains									
VSSA (NRS149)	++	++	++	+++	+++	+	+	++	++
hVISA (Mu3)	−/+	−/+	−/+	++	++	−/+	−/+	−	−
VISA (Mu50)	−	−	−	−	−	−	−	−	−
agr knockout (NRS155)	−	−	−	−	−	−	−	−	−
Clinical isolates									
VSSA ($n = 8$)	8	8	8	8	8	8	8	8	8
hVISA ($n = 24$)	22	20	20	19	17	21	19	19	22
NARSA VISA ($n = 5$)	4	4	4	4	4	4	4	4	5
Total no. of VISA isolates correctly detected	26	24	24	23	21	25	23	23	27
Sensitivity (%)	89.6	82.7	82.7	79.3	72.4	86.2	79.3	79.3	93.1
Specificity (%)	100	100	100	100	100	100	100	100	100

[a] For the prototype strains, the δ-hemolysis zone size is shown. For the clinical isolates, the numbers of hVISA/VISA strains correctly identified by the different 5% sheep blood media tested are shown.

[b] OX COL, 5% sheep blood agar with Columbia base from Oxoid; BM COL, 5% sheep blood agar with Columbia base from bioMérieux; BD COL, 5% sheep blood agar with Columbia base from Becton Dickinson; OX MH, 5% sheep blood agar with Mueller-Hinton base from Oxoid; BM TSA, 5% sheep blood agar with Trypticase soy agar base from bioMérieux; BD TSA, 5% sheep blood agar with Trypticase soy agar base from Becton Dickinson; HM and LC COL VAN, 5% sheep blood agar with Columbia base and 6 mg/liter VAN, with the agar being homemade and Liofilchem produced, respectively.

medium gave a negative result, resolving discrepancies and giving a more precise interpretation of the δ-hemolysis assay for hVISA (Fig. 1).

For all tested media, comparing the δ-hemolysis zone of the sample with those of control strains and consequently establishing the number of strains that were correctly categorized as VSSA, hVISA, and VISA (according to PAP analysis), we calculated the sensitivity and specificity as shown in Table 1.

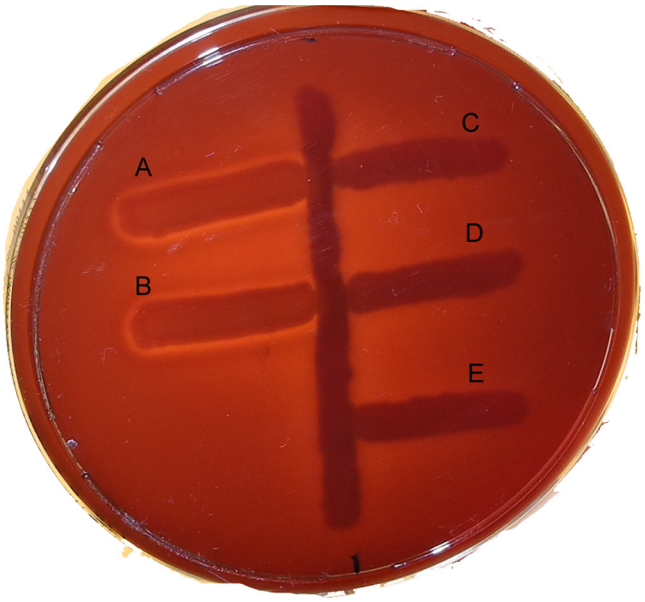

FIG 1 δ-Hemolysis assays on 5% Columbia sheep blood agar with 6 mg/liter VAN. (A) NRS149 (VSSA prototype); (B) VSSA clinical isolate; (C) Mu3 (hVISA prototype); (D) Mu50 (VISA prototype); (E) NRS155 (*agr* knockout strain).

In particular, HM and LC COL with 6 mg/liter VAN sheep blood agar showed the highest sensitivity (93.1%) followed by OX COL sheep blood agar (89.6%), whereas the other tested media showed sensitivity of less than 86.2%. Comparing the sensitivity and specificity of the HM and LC COL with 6 mg/liter VAN sheep blood agar with those obtained with vancomycin and teicoplanin by a Macro Etest (2 McFarland standard [2McF]) in previously published data (4), it emerged that the sensitivity of HM and LC COL with 6 mg/liter VAN was 93.1% and that of OX COL was 89.6%, whereas vancomycin and teicoplanin Macro Etest sensitivity was 75%. The specificity was 100% for all media tested, the same as that found for the vancomycin and teicoplanin Macro Etest (4).

To support our δ-hemolysin data, we analyzed the *hld* mRNA amount (δ-hemolysin-encoding gene) in two *agrI* clinical hVISA strains (61D, P3), five *agrII* clinical hVISA strains (CZ1, 004/210, AN9, SS33, AN4), three *agrII* NARSA VISA strains (NRS3, NRS4, NRS404), three reference *agrII* strains (NRS149, Mu3, Mu50), and a negative control (NRS155) by relative quantitative real-time reverse transcription PCR (real-time RT-PCR).

Bacterial cultures, RNA extraction, and cDNA synthesis were carried out as previously published (2, 3). Quantitative real-time RT-PCR was performed with the following primers: *gyr*B-up, 5′-CAACTATGAAACATTACAGCAGCGT-3′; *gyr*B-down, 5′-TGTGGCATATCCTGAGTTATATTGAAT-3′; *hld*-up, 5′-CTGAGTCCAAGGAAACTAACTCTAC-3′; *hld*-down, 5′-TGATTTCAATGGCACAAGAT-3′. PCR efficiency was verified as previously published and *gyrB* was used as a normalizer (2). *hld* expression is represented as fold changes of hVISA/VISA toward VSSA. Five distinct biological replicates were used.

Statistical expression analyses were performed using the REST2009 (Relative-Expression-Software-Tool) (http://rest.gene-quantification.info/), and differences were considered significant at a P value of <0.05 (9, 10).

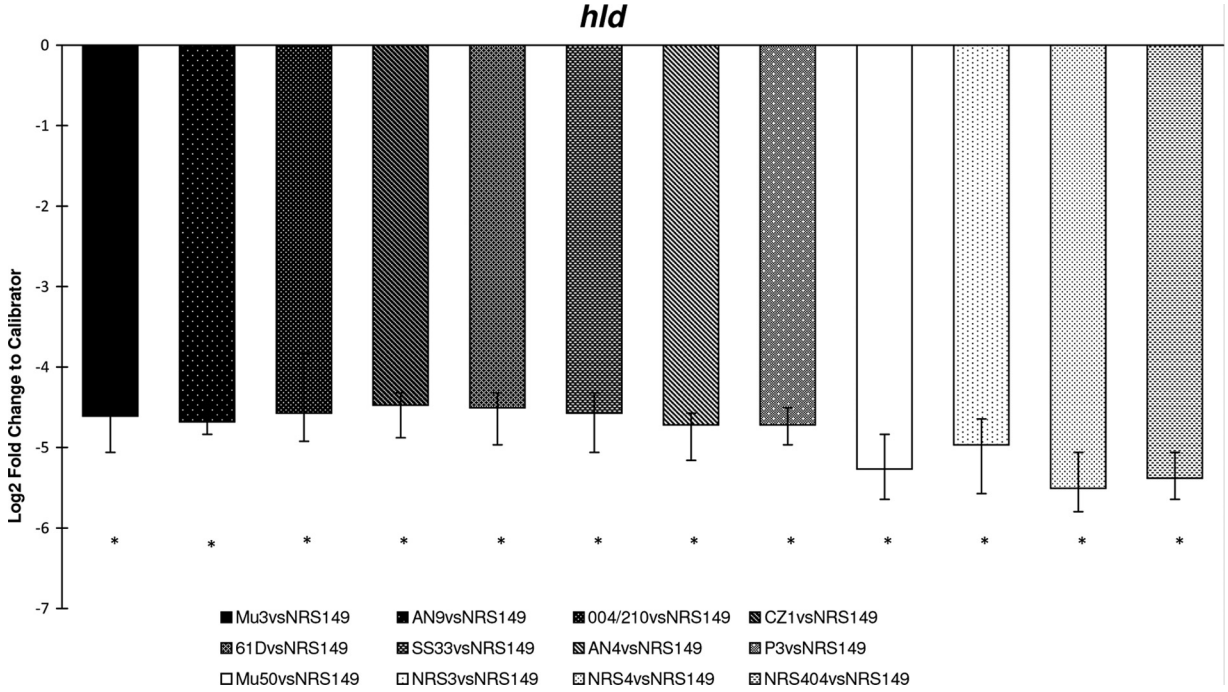

FIG 2 Relative quantitative expression of *hld* in hVISA and VISA. Darker bars indicate hVISA; lighter bars indicate VISA. *, statistically significant differences between isolates and VSSA (NRS149) ($P < 0.05$).

The results of real-time RT-PCR, correlating with data obtained from the phenotypic assays of δ-hemolysin production, showed a gradual and substantial *hld* downregulation, independently from a specific *agr* group membership, as follows: VSSA>hVISA>VISA (Fig. 2). NRS155, as expected, presented no *hld* amplification (no threshold cycle [C_T] value).

Thus, we propose the use of the δ-hemolysin assay in COL 5% sheep blood agar with 6 mg/liter of vancomycin as a clear and rapid screening method to accurately detect hVISA/VISA strains. We found that the addition of vancomycin resolves any misinterpretations, increasing the sensitivity of the method. A limitation of the study is the reduced number of clinical VISA isolates tested (the isolates are still rare); however, our data show that this can be a rapid, cheap, highly sensitive, and specific method to screen hVISA in clinical practice.

ACKNOWLEDGMENTS

We are grateful to Antony Bridgewood for the language revision and to NARSA for sending NARSA strains.

This study was funded by a grant from the Italian Minister of Health, MIUR-PRIN 20087SM5HM.

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