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NOTES

Multiplex PCR-Ligation Detection Reaction Assay for Simultaneous Detection of Drug Resistance and Toxin Genes from *Staphylococcus aureus*, *Enterococcus faecalis*, and *Enterococcus faecium*▽†

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A multiplex PCR-ligation detection reaction (PCR-LDR) assay was developed for rapid detection of methicillin, tetracycline, and vancomycin resistance, as well as toxic shock toxin and Panton-Valentine leukocidin. The assay was tested on 470 positive blood culture bottles containing *Staphylococcus aureus* or enterococci. PCR-LDR exhibited a sensitivity and specificity of ≥98% for all components except tetracycline resistance, which had a sensitivity of 94.7%. Rapid and sensitive detection of antimicrobial resistance and virulence genes could help guide therapy and appropriate infection control measures.

Previous studies have demonstrated the ability of multiplex PCR-ligation detection reaction (PCR-LDR) assays to detect and identify a variety of clinically significant bacteria and flaviviruses (4, 12, 14). We have now developed a multiplex PCR-LDR assay to directly determine antibiotic resistance profiles, as well as the toxic shock syndrome (Tsst-1) and Panton-Valentine leukocidin (PVL) toxins, of *Staphylococcus aureus*, *Enterococcus faecalis*, and *Enterococcus faecium* present in positive blood culture bottles. Rapid identification of antimicrobial resistance and virulence determinants could have a significant impact on reducing morbidity, mortality, and health care costs. Methicillin and vancomycin resistance genes were included in the assay because of the clinical importance of vancomycin-resistant enterococci (VRE), methicillin-resistant *S. aureus* (MRSA), and most recently vancomycin-resistant *S. aureus* (VRSA) infections (5, 15, 18). Tetracycline resistance genes were included because of renewed interest in this group of antibiotics due to their activity against several biothreat agents and the increase in community-acquired MRSA (7, 8, 21, 22).

Positive blood cultures containing *S. aureus*, *E. faecalis*, and/or *E. faecium* were obtained from patients at Weill Cornell Medical Center, New York Presbyterian Hospital (WCMC-NYPH). Blood cultures were collected and incubated in a BacT/Alert system (bioMérieux, Durham, NC) in accordance with the manufacturer's instructions. When a blood culture was read as positive by the BacT/Alert, 100-μl aliquots of the blood culture were transferred in quadruplicate to a 96-deep-well plate for subsequent extraction of DNA; negative controls containing only Tris-EDTA buffer were incorporated into each 96-well plate, and the plates were stored at −70°C. An additional 400 μl of the positive blood culture was stored for retesting of any discordant results. Bacteria were identified using standard methods. Additional clinical isolates of *S. aureus*, *E. faecalis*, and *E. faecium* were collected from wound, urine, respiratory, or autopsy samples. These isolates were spiked into negative clinical blood culture bottles as described previously (12). The use of human clinical samples in this research has complied with all relevant federal guidelines and WCMC-NYPH institutional policies.

Bacterial isolates previously shown by sequencing to contain the *mecA*, *vanA*, *vanB*, *tetK*, *tetL*, *tetM*, or *lukS-lukF* (PVL) gene(s) were obtained from the WCMC-NYPH clinical microbiology collection. *E. faecium* N97-0330 (*vanD3*) and *E. faecium* N03-0072 (*vanD5*) (1) were provided by Michael Mulvey. *E. faecium* NEF1 (*vanD1*) (9) was provided by Albert Sotto and Jean Phillippe Lavigne.

DNA from clinical specimens was extracted using an ABI 6100 nucleic acid prep system (Applied Biosystems, Foster City, CA) as described previously (12). The procedure used fine grade charcoal to remove sodium polyanetholesulfonate (SPS), an essential component of blood culture media and a potent inhibitor of PCR.

PCR and LDR primers were designed based on sequences obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) as previously described (12). In instances where sequences did not share 100% identity, multiple PCR/LDR

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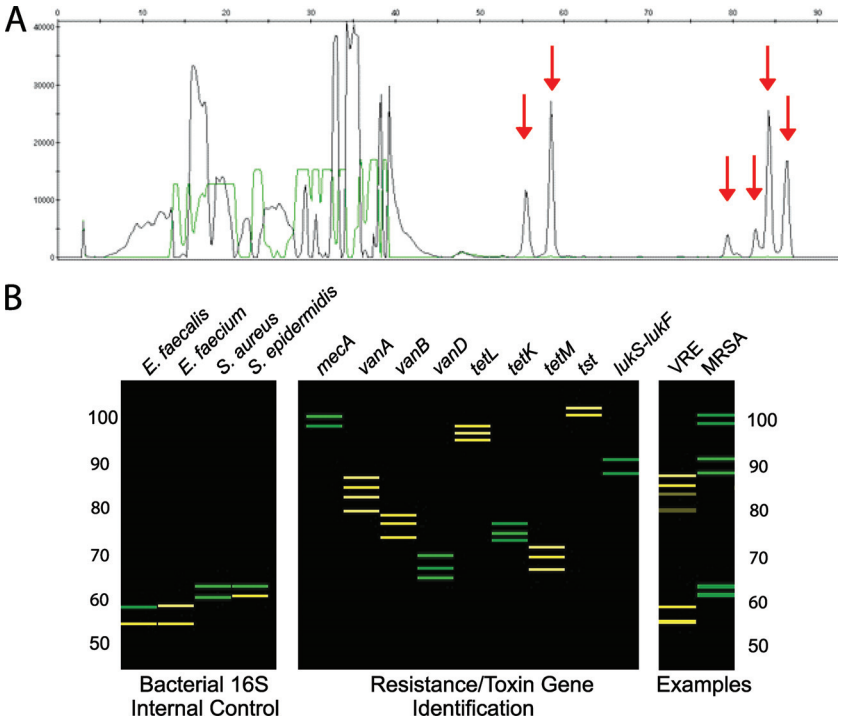

FIG. 1. (A) Capillary electrophoresis (CE) trace for the antibiotic resistance, and toxin PCR-LDR assay of a vancomycin-resistant *E. faecium* isolate. The *x* axis shows the LDR product size, and the *y* axis shows fluorescence intensity of the bands. Red arrows indicate signals that are unique to the 16S rRNA genes of *E. faecium* and the *vanA* gene. (B) The CE data are displayed as a reconstructed gel image generated by a software program developed in our laboratory. The left panel represents the size and fluorescence of the 16S rRNA gene LDR products which act as a positive control and differentiate between the bacteria *E. faecalis*, *E. faecium*, and *Staphylococcus* species. The middle panel represents the LDR signals that identify the presence of methicillin resistance (*mecA*), vancomycin resistance (*vanA*, *vanB*, *vanD*), tetracycline resistance (*tetL tetK*, *tetM*), the toxic shock toxin gene (*tst*), and the PVL toxin genes (*lukS-lukF*). Data in the right panel represent two blood cultures. In one, the 16S rRNA gene control identified *E. faecium*, and the *vanA* gene was detected, indicating a VRE; in the other the 16S rRNA gene control identified *S. aureus*, and the *mecA* and *lukS-lukF* genes were detected, indicating an MRSA gene encoding the PVL toxin.

primers were designed over the selected region. To prevent failure of PCR amplification, two PCR primer pairs were designed for each of the gene amplicons. The 44 PCR primers used in the multiplex PCR assay are presented in Table SA1 in the supplemental material. The 71 LDR primers used in the multiplex LDR assay are presented in Table SA2 in the supplemental material. Two to three LDR primer pairs were designed for each of the PCR amplicons. Identification of at least two LDR signals for any gene was required for a positive result. The assay also incorporated two previously defined positive amplification controls specific to the 16S rRNA genes, identifying the bacteria as *Staphylococcus* species, *E. faecium*, or *E. faecalis* (12). PCRs and LDR were performed as described in Table SA3 in the supplemental material. Capillary electrophoresis (CE) was performed and analyzed as previously described (4, 12, 14) (Fig. 1).

The antibiotic susceptibility of each organism was determined by VITEK2 (bioMerieux, Durham, NC). If an intermediate or discrepant call was made by the VITEK2, secondary/confirmatory testing was performed by Etest (AB Biodisk, Solna, Sweden). MICs were interpreted based on CLSI guidelines (3). Production of Tsst-1 was confirmed by reverse passive latex agglutination, using the TST-RPLA kit (Oxoid, United Kingdom). *S. aureus* ATCC 51651 and ATCC 13566 were used as positive and negative controls, respectively. The presence of the PVL toxin genes was confirmed by amplification and sequencing by using the primers luk-PV-1 and luk-PV-2 (10).

The resistance and toxin genotypes of 470 blood culture samples (193 positive blood cultures and 277 clinical isolates spiked into negative blood cultures) were examined using the multiplex PCR-LDR assay. Twenty of the positive blood cultures were polymicrobial; fourteen of these contained at least one *S. aureus*, *E. faecium*, or *E. faecalis* resistance gene.

Correlation of the multiplex PCR-LDR assay with standard susceptibility testing for pure cultures is presented in Table 1. The PCR-LDR assay detected methicillin resistance in *S. aureus* with 99.2% sensitivity and 99.1% specificity. One isolate was *mecA* positive and oxacillin susceptible, and one was *mecA* negative and oxacillin resistant. Vancomycin resistance was detected in *E. faecium* and *E. faecalis* with 98.6% sensitivity and specificity compared to susceptibility testing. Almost all (98.4%) vancomycin resistance in *E. faecium* was due to *vanA*, whereas *vanB* was responsible for an appreciable portion (44.4%) of resistance in *E. faecalis* (see Table SA4 in the supplemental material). Two isolates of *E. faecalis* were vancomycin susceptible but were positive for *vanA* or *vanB*. Low-level expression of the *van* genes may have failed to raise the MIC above the breakpoint for resistance. One isolate of *E. faecium* was vancomycin resistant but was negative for *vanA*, *vanB*, and *vanD*. Re-

TABLE 1. Antibiotic resistance phenotypes and PCR-LDR gene identification in blood cultures containing *S. aureus* or enterococci

Phenotype[a]	No. of samples	No. of organisms with indicated genotype	
		mecA positive	*mecA* negative
MRSA	124	123	1
MSSA	111	1	110
		vanA or *-B* positive	*vanA* or *-B* negative
E. faecium Van^r	64	63	1
E. faecium Van^s	14	0	14
		vanA or *-B* positive	*vanA* or *-B* negative
E. faecalis Van^r	9	9	0
E. faecalis Van^s	128	2	126
		tetK, *-L*, or *-M* positive	*tetK*, *-L*, or *-M* negative
S. aureus Tet^r	22	22	0
S. aureus Tet^s	213	2	211
E. faecalis Tet^r	105	99	6
E. faecalis Tet^s	32	0	32
E. faecium Tet^r	15	13	2
E. faecium Tet^s	63	1	62

[a] r, resistance; s, susceptibility; Van, vancomycin; Tet, tetracycline.

TABLE 2. Antibiotic resistance phenotypes and PCR-LDR gene identification in mixed cultures

Organism identification and resistance phenotype[a]	PCR-LDR gene identification
E. faecalis Tet^r, *E. faecium*	*tetM*
E. faecalis, *E. faecium* Van^r	*vanA*
E. faecalis Tet^r, *E. faecium* Van^r	*tetM vanA*
E. faecalis Tet^r Van^r, *E. faecium*	*tetM vanA*
E. faecalis, *E. faecium* Tet^r Van^r, CNStaph	*tetM vanB*
E. faecalis Tet^r, MRSA Oxa^r	*tetM mecA*
E. faecalis Tet^r, MRSA Oxa^r	*tetM mecA*
E. faecalis, *S. aureus*, *K. pneumoniae*[b]	*tetM*
E. faecalis Tet^r, *S. aureus*, *K. pneumoniae*	*tetM*
E. faecalis Tet^r, CNStaph[c]	*tetL*
E. faecalis Tet^r, CNStaph	*tetL tetM*
E. faecalis Tet^r, CNStaph[c]	*tetM*
E. faecalis Tet^r Van^r, CNStaph[c]	*tetM vanA*
E. faecium Van^r, MRSA Oxa^r	*vanA mecA*

[a] There were six other mixed cultures that did not contain resistant organisms. CNStaph, coagulase-negative staphylococcus; Oxa, oxacillin.

[b] *E. faecalis* and *S. aureus* were phenotypically Tet^s. *K. pneumoniae* was not tested.

[c] These CNStaph isolates were oxacillin resistant, but PCR-LDR did not detect *mecA* or staphylococcal 16S rRNA gene sequences, presumably due to small numbers of organisms.

sistance may have been due to gene sequences not incorporated into this assay, such as *vanE* or *vanG* (5).

Tetracycline resistance was detected in staphylococci and enterococci with 94.6% sensitivity and 99.0% specificity compared to standard susceptibility testing. The assay detected resistance in 22 isolates of *S. aureus* with 100.0% sensitivity and 99.0% specificity. The mechanism of resistance was divided between efflux pumps (*tetK*, 68.2%) and ribosomal protection proteins (*tetM*, 31.8%) (see Table SA4 in the supplemental material). In contrast, 98.2% of the resistance in *E. faecium* and *E. faecalis* was due to ribosomal protection proteins (*tetM*). Two *S. aureus* and one *E. faecium* were positive for *tetK* or *tetM* but susceptible in the phenotypic assay. This may result from low-level expression of the *tet* genes. Eight enterococci were tetracycline resistant but negative for *tetK*, *tetL*, and *tetM*. This is likely due to tetracycline resistance conferred by other gene sequences not incorporated into this assay (e.g., *tetO*, *tetQ*, or *tetS*) (13).

Table 2 shows the antibiotic phenotypes and PCR-LDR results from mixed cultures. Although the resistance genes detected by the assay could not be precisely assigned to a specific organism because of the ability of staphylococci and enterococci to harbor these resistance elements, the PCR-LDR assay was able to identify and detect the predicted resistance genes for each mixed culture.

The *tst* gene encoding the Tsst-1 toxin was identified by the multiplex PCR-LDR assay in 15/242 samples containing *S. aureus* (6.2%; nine methicillin-susceptible *S. aureus* [MSSA] and six MRSA isolates). Tsst-1 activity was confirmed in 14 of the positive samples by using the TST-RPLA kit (one sample failed to grow from the archived material). Lack of Tsst-1 activity was confirmed in 25/25 *S. aureus* samples randomly selected from those organisms that were negative for the *tst* gene. The *lukS* and *lukF* PVL genes were detected in 24.0% of MRSA and 7.0% of MSSA samples. There was a higher incidence of PVL in non-bloodstream-infection (non-BSI) MRSA samples (27.0%) than that in BSI MRSA samples (18.0%).

Several real-time PCR assays have been developed that can detect *S. aureus* and MRSA in positive blood cultures (17, 23). A multiplex assay utilizing bead hybridization was able to distinguish several species of staphylococci and detect PVL, *mecA*, and other resistance genes (20). PCR-LDR assays detected methicillin and vancomycin resistance in *S. aureus*, *E. faecalis*, and *E. faecium* with >98% sensitivity and specificity compared to phenotypic susceptibility testing. The specificity may be an underestimate, since in several situations, such as determining methicillin resistance, gene detection is the more appropriate gold standard (3). The lower sensitivity we observed for detecting tetracycline resistance is probably due to our assay targeting only 3 of 38 known *tet* genes. Even large-scale multiplex assays might need to be restricted to detecting resistance to a limited number of "front-line" antibiotics. Our data also illustrate the difficulty of interpreting genotype data from mixed cultures; nonetheless, detection of resistance genes in these specimens could help guide empirical antibiotic therapy.

Tsst-1 and PVL toxins are commonly associated with community-acquired strains of *Staphylococcus aureus* (10); however, not all community-acquired strains carry the PVL toxin, and Tsst-1 can be found in nosocomial strains (2, 15, 16). Community-acquired MRSA strains, which are more likely to have these virulence genes, are more commonly found in skin or soft-tissue infections than in bloodstream infections (8, 11, 15, 16). Of the 15 isolates carrying *tst* in our study, 73% came from non-BSIs. Similarly, of the 45 isolates carrying PVL toxin genes, 76% were from non-BSIs, and 71% were methicillin resistant. Although these virulence factors are not stable markers for distinguishing between community-acquired and nosocomial *Staphylococcus* strains, they are nevertheless important for molecular epidemiology and monitoring virulence factors and antibiotic resistance patterns. Combined with conventional culturing methods, a multiplex molecular assay has the potential to provide accurate, timely information, resulting in improved

patient care and a reduction in broad-spectrum antibiotic usage while also providing epidemiological data for infection control and antimicrobial/toxin surveillance systems (6, 19).

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