JOURNAL OF CLINICAL MICROBIOLOGY, July 2008, p. 2377–2380 Vol. 46, No. 7
0095-1137/08/$08.00+0 doi:10.1128/JCM.00230-08

Optimization of a Laboratory-Developed Test Utilizing Roche Analyte-Specific Reagents for Detection of *Staphylococcus aureus*, Methicillin-Resistant *S. aureus*, and Vancomycin-Resistant *Enterococcus* Species▽

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Received 4 February 2008/Returned for modification 21 March 2008/Accepted 24 April 2008

Nasal and perianal swab specimens were tested for detection of *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* species (VRE) using a laboratory-developed real-time PCR test and microbiological cultures. The real-time PCR and culture results for *S. aureus* were similar. PCR had adequate sensitivity, but culture was more specific for the detection of VRE.

Staphylococcus aureus, especially methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant *Enterococcus* species (VRE) are important pathogens associated with health care facility outbreaks worldwide (1, 2, 8, 21). It has been reported that identifying asymptomatically colonized individuals and placing them into contact isolation within a short time frame are a good management tool for reducing the spread of these pathogens and for lowering health care-associated infections (5, 11, 16, 18). Furthermore, there is rising interest in identifying all *S. aureus* carriers prior to surgery to decolonize them to reduce postoperative *S. aureus* surgical-site infections (4, 10).

Reliable surveillance requires accurate testing (17), and cultures can take from 2 to 5 days to obtain the final results (3a). PCR offers detection of *S. aureus* and VRE directly from swab specimens within a few hours (4, 12, 14) and can help with the rapid deployment of infection control and prevention measures (16, 18).

The purpose of our study was to develop an optimized test using Roche analyte-specific reagents (ASRs) for detection of *S. aureus* and VRE by real-time PCR with a single set of amplification conditions and to compare the PCR results to the results of conventional culture.

Inpatients at Evanston Northwestern Healthcare during August 2004 made up the patient population. Premoistened, double-headed swabs (culture swab; BBL, Becton Dickinson, Franklin Lakes, NJ) were used to collect paired anterior nasal specimens and paired perianal specimens as part of an infection control activity to determine colonization prevalence. There were 387 nasal specimens (for *S. aureus*) and 309 perianal specimens (for VRE). This investigation was approved by the Institutional Review Board of Evanston Northwestern Healthcare.

For *S. aureus*, one of the paired nasal swabs was plated onto Columbia colistin-nalidixic agar with 5% sheep blood (Remel, Inc., Lenexa, KS) (3a) and incubated at 35°C for 48 h. *S. aureus* was identified by colony morphology and Staphaurex latex agglutination testing (Remel, Inc.). Methicillin resistance was determined on colonies by using PCR as described below. For any swabs that were PCR positive only for *S. aureus* ($n = 13$), the original cultures were reexamined beyond their initial 48-hour incubation, with five additional swab samples yielding *S. aureus* after reexamination (two yielded MRSA).

VRE was cultured by plating one of the paired perianal swabs on bile esculin azide agar containing 6 μg of vancomycin/ml agar (Remel, Inc.) and by incubation at 35°C for 72 h. Colonies black in color (bile esculin positive) were confirmed to be *Enterococcus faecium* or *Enterococcus faecalis* by using conventional biochemical testing. Vancomycin resistance was determined via Etest in accordance with the Clinical and Laboratory Standards Institute guidelines (3). For any samples that were PCR positive only, the original swabs were placed into thioglycolate broth (BBL, Becton Dickinson) and incubated for 72 h at 35°C. The broth was then subcultured onto a colistin-nalidixic agar plate; 2 of 22 specimens grew VRE.

Although the swab processing and extraction methods were unique for each assay, we designed identical real-time PCR amplification conditions for all three assays that are presented in Table 1. This was done to facilitate the running of samples for various assay targets at the same time on the LightCycler instrument. The second swab from each surveillance specimen was broken off in a microcentrifuge tube and processed. For *S. aureus*, after the incubation steps, fluid surrounding the swab was aspirated and directly used for real-time PCR analysis (12). For VRE, by using the extraction protocol summarized in Table 1, a final eluate of 100 μl of purified DNA was used for real-time PCR. All ASRs were supplied by Roche Diagnostics.

Each culture that grew an *S. aureus* isolate was tested for the presence of *mecA* from colonies using the Roche LightCycler

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▽ Published ahead of print on 30 April 2008.

TABLE 1. Real-time PCR protocols for using the Roche ASRs to detect S. *aureus* and VRE on the LightCycler instrument

Preparation step and run conditions	Protocol to detect:		
	S. aureus	*mecA*	VRE (*vanA*, *vanB*, and *vanB2/3* genes)[a]
Specimen	Nasal swab	Bacterial colonies	Perianal swab
Extraction	Swab broken off into a microcentrifuge tube containing 200 μl of a 1-U/μl achromopeptidase solution	2 or 3 isolated colonies placed into a microcentrifuge tube with 1% Triton X-100, 0.5% Tween 20, 1 mmol/liter Tris-HCl (pH 8.0), and 10 mmol/liter EDTA	Swab broken off into a microcentrifuge tube containing 100 μl of STAR buffer (Roche)
Processing	Tube vortexed for 3–5 s and then incubated at 37°C for 15 min, followed by 5 min of incubation at 100°C	Tube incubated at 100°C for 10 min and then centrifuged for 1 min at >10,000 × *g*	Tube processed in accordance with protocol for MagNA Pure LC microbiology kit M^{GRADE}, specimens extracted on MagNA Pure LC using the DNA M^{GRADE} protocol
Reaction mix	2–5 μl of extracted DNA, 2 μl of LightCycler FastStart DNA master hybridization probe M^{GRADE} mix, 2 μl of LightCycler *Staphylococcus* M^{GRADE} primer/hybridization probes, 1 μl of 1:10 dilution of LightCycler *Staphylococcus* M^{GRADE} recovery template, 10 μl of sterile water	2 μl of extracted DNA, 2 μl of LightCycler FastStart DNA master hybridization probe M^{GRADE} mix, 2 μl of LightCycler *mecA* primer/hybridization probes, 2 μl of LightCycler *mecA* recovery template, 2.4 μl of $MgCl_2$, 9.6 μl of sterile water	5 μl of extracted DNA, 2 μl of LightCycler FastStart DNA master hybridization probe M^{GRADE} mix, 2 μl of LightCycler *vanA*/*vanB* primer/hybridization probes, 2 μl of LightCycler *vanA*/*vanB* recovery template, 2 μl of $MgCl_2$, 7 μl of sterile water
Controls	LightCycler *Staphylococcus* M^{GRADE} template set was the positive control; sterile water was the negative control	LightCycler *mecA* template DNA was the positive control; sterile water was the negative control	LightCycler *vanA*/*vanB* template set was the positive control; sterile water was the negative control
Real-time PCR conditions	Initial step of 10 min at 95°C, followed by amplification for 45 cycles of 10 s at 95°C, 10 s at 55°C, and 12 s at 72°C, with fluorescence acquisition at the end of each annealing	Initial step of 10 min at 95°C, followed by amplification for 45 cycles of 10 s at 95°C, 10 s at 55°C, and 12 s at 72°C, with fluorescence acquisition at the end of each annealing	Initial step of 10 min at 95°C, followed by amplification for 45 cycles of 10 s at 95°C, 10 s at 55°C, and 12 s at 72°C, with fluorescence acquisition at the end of each annealing
Melt program	Ramp to 95°C, followed by 20 s at 59°C, 20 s at 45°C at a rate of 0.2°C/s, and a gradual increase to 85°C at a rate of 0.2°C/s with continuous fluorescence acquisition	Ramp to 95°C, followed by 20 s at 59°C, 20 s at 45°C at a rate of 0.2°C/s, and a gradual increase to 85°C at a rate of 0.2°C/s with continuous fluorescence acquisition	Ramp to 95°C, followed by 20 s at 59°C, 20 s at 45°C at a rate of 0.2°C/s, and a gradual increase to 85°C at a rate of 0.2°C/s with continuous fluorescence acquisition

[a] STAR, stool transport and recovery.

Staphylococcus and LightCycler *mecA* ASRs in an in-house real-time PCR assay (13). For DNA extraction, two or three isolated colonies of *S. aureus* were touched with a sterile loop, placed into a microcentrifuge tube containing lysis buffer, and processed (Table 1). The PCR results were assessed using culture results as the reference standard. The chi-square statistic was used for determining any significant difference.

The results of our study are shown in Table 2. Eleven specimens were culture positive and PCR negative, of which two were MRSA. Six of these 11 specimens had only one to three *S. aureus* colonies, indicating very low density colonization, and thus those negative specimens were likely below the detection sensitivity for the PCR assay.

Colonies from each culture that grew *S. aureus* were tested for the presence of *mecA* by using the Roche LightCycler *mecA* ASR and our in-house real-time PCR assay (13). The *mecA* colony PCR results were identical for both methods. Out of a total of 105 *S. aureus* specimens, 33 specimens (31%) were *mecA* positive (MRSA).

The laboratory-developed VRE real-time PCR assay detects *vanA*, *vanB*, and *vanB2/3* genes and differentiates them by using melt curve analysis (Table 2). Melt curve analysis of the 15 culture- and PCR-positive swabs showed that 11 specimens had the *vanA* gene alone, one contained the *vanA* and *vanB* genes, one had the *vanB2/3* gene (considered a false-positive test), and two had the *vanA* and *vanB2/3* genes. The PCR results for the cultured colonies showed only the presence of *vanA* in all 15 specimens. For the four swab specimens that were culture positive and PCR negative for VRE, all four isolates were *E. faecium* with MICs of 8, 16, 16, and >256 μg/ml. The PCR results for these colonies found that only one harbored the *vanA* gene while the other three tested negative; a determinant for vancomycin resistance in these is unknown. For the 20 swab specimens that were culture negative and PCR

TABLE 2. Results of real-time PCR testing for *S. aureus*[a] and VRE[b] from surveillance swabs with culture used as the reference standard

Organism	No. of true positives	No. of true negatives	No. of false positives	No. of false negatives	% Sensitivity (95% CI)	% Specificity (95% CI)	% Positive predictive value (95% CI)	% Negative predictive value (95% CI)
S. aureus (n = 106 positive samples)[c]	95	270	8	11	89.6 (81.8–94.5)	97.1 (94.2–98.7)	92.2 (84.8–96.3)	96.1 (92.9–97.9)
VRE (*vanA*, *vanB*, and *vanB2/3* genes; n = 19 positive samples)[d]	14	246	21	5	73.7 (48.6–89.9)	92.1 (88.1–94.9)	40 (24.4–57.8)	98 (95.2–99.3)
VRE (*vanA* gene only; n = 16 positive samples)[c]	14	268	2	2	87.5 (60.4–97.8)	99.3 (97.1–99.9)	87.5 (60.4–97.8)	99.3 (97.1–99.9)

[a] Ten specimens (2.6%) were inhibited in the real-time PCR test when 5 μl of DNA was used. These were retested using 2 μl of DNA, and seven gave an amplification result, with six being negative and one positive for *S. aureus*, matching the culture results. Three specimens remained inhibited and were excluded from the data analysis, giving a total of 384 analyzed specimens.

[b] Eighteen specimens were inhibited (5.8%), and for another 5 specimens, the MagNA Pure LC instrument malfunctioned, with no DNA being extracted, and thus these specimens were not included in the final analysis, giving a total of 286 analyzed specimens.

[c] Difference between PCR and culture results, the P value was not significant.

[d] The difference between PCR and culture results was significant at a P value of 0.005.

positive, 2 had *vanA*, 4 had *vanB*, and 14 had *vanB2/3*, as determined by the PCR assay.

The costs per test (based on manufacturers' suggested retail price) plus operator time for processing were $4.00 to $4.50 plus 1 to 2 min for culture, $3.00 plus 2 to 3 min for in-house (*S. aureus* and *mecA*) PCR, and $21.00 (*S. aureus* and *mecA*) to $42.00 (VRE) plus 2 to 3 min for the commercial ASR tests we describe.

Both the *S. aureus* and VRE real-time PCR assays yielded results within 3 to 5 h, including extraction and assay run time, compared to culture, which took from 48 to over 72 h. While new chromogenic agar can detect MRSA with overnight incubation, the sensitivity of direct testing is <80%, compared to PCR (15a). The ASR reagents investigated gave reliable results for detection of *S. aureus*; however, confirmation of MRSA by detection of *mecA* required growth of *S. aureus* colonies. We have demonstrated that the use of such a testing strategy to detect *S. aureus* in presurgical patients can significantly lower their risk of postoperative *S. aureus* infections (4).

In our VRE testing, we found that while PCR assay of the swab detected *vanA*, *vanB*, and *vanB2/3* genes, PCR confirmation using the recovered colonies only detected *vanA*. The 17 specimens with the *vanB2/3* signal may represent a false-positive amplification test for VRE. The *vanB* gene is known to occur in other bacteria (21); however, *vanB2/3* containing enterococci can potentially cause outbreaks (7, 9), so this result cannot be ignored. Also, we have previously shown that PCR-positive surveillance swabs for both *S. aureus* and VRE can indicate persons harboring these pathogens at other sites or prior times (12, 14, 15, 20), so some of the PCR-positive results may represent false-negative cultures. Based on our findings, and those of others (6), the VRE real-time PCR test in a clinical setting appears good for early detection of patients likely infected with VRE; however, it would seem prudent to culture the specimens that signal positive for *vanB* and *vanB2/3* in order to confirm that those patients indeed harbor VRE, as without such confirmation the positive predictive value of the test is only 40%.

Sloan and colleagues (19) found better sensitivity (100%) that we did and a similar specificity when using the Roche LightCycler *vanA/vanB* ASRs in their laboratory. One of the reasons for higher sensitivity may be that they used a different culture method (19). Also, only PCR-positive samples were evaluated for the presence of VRE colonies.

We have optimized laboratory-developed tests that utilize commercially available ASRs that can detect *S. aureus* (and that can confirm MRSA after culture) and VRE directly from surveillance swab specimens by using a single set of amplification conditions. These tests can help clinicians make important infection control and surgical prophylaxis decisions. The VRE real-time PCR test using currently available ASR materials reliably detected the VanA phenotype but detected many more *vanB* and *vanB2/3* positives than culture, suggesting that this latter result may need to be confirmed by culture.

This work was supported by a research grant from Roche Diagnostics Corporation, Indianapolis, IN, to Lance R. Peterson. There are no other potential conflicts of interest for this report for any of the other authors.

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