Accuracy and Potential Usefulness of Triplex Real-Time PCR for Improving Antibiotic Treatment of Patients with Blood Cultures Showing Clustered Gram-Positive Cocci on Direct Smears

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Bacterial identification and antibiotic susceptibility testing currently require 48 h when a first blood culture (BC) is positive for clustered gram-positive cocci on direct smear examination (DSE). Meanwhile, antibiotic treatment is often inadequate, reducing the chances of effective treatment or creating unnecessary selective pressure. A new real-time PCR (RT-PCR) technique that differentiates Staphylococcus aureus from coagulase-negative staphylococci (CoNS) and detects methicillin resistance in 90 min in BC bottles could help solve these problems. BC bottles from 410 patients with gram-positive cocci on DSE were processed by current methods, and patients’ treatments were prospectively recorded. The RT-PCR assay was performed on aliquots of these BCs, which had been kept frozen. For the 121 patients who had true bacteremia, we established whether the faster availability of RT-PCR results could have led to the initiation of treatments different from those actually given. RT-PCR sensitivity and specificity were 100% for differentiating between S. aureus and CoNS and detecting methicillin resistance with two manufacturers’ BC bottles. For 31/86 (36%) of the S. aureus-infected patients and for 8/35 (23%) of the CoNS-infected patients who either had suboptimal or nonoptimal treatment or were untreated 48 h after positivity was detected, the early availability of RT-PCR results could have allowed more effective treatment. Unnecessary glycopeptide treatments could have been avoided for 28 additional patients. The use of RT-PCR would increase treatment effectiveness in patients with staphylococcal bacteremia and reduce the selective pressure created by glycopeptides.

Bacterial bloodstream infections (BBSI) are a leading cause of morbidity, mortality, and increased health care costs (31). Staphylococci are the second most prevalent bacteria (46). However, a smear suggesting the presence of staphylococci in a blood culture (BC) is not sufficient for the diagnosis of true BBSI before the species is identified, because coagulase-negative staphylococci (CoNS) are part of the normal skin flora and may contaminate BC bottles during venous puncture procedures. Therefore, the presence of CoNS in BCs is not usually considered clinically significant unless two or more cultures are positive (48). By contrast, because Staphylococcus aureus is intrinsically virulent, its isolation in only one BC bottle is usually enough to diagnose BBSI and initiate antibiotic therapy (18).

Since the first appearance of methicillin-resistant S. aureus (MRSA) strains in 1960, they have become a serious problem worldwide (14). BBSI due to MRSA may account for up to 30% of all S. aureus BBSI (9). However, vancomycin, the antibiotic treatment of choice for MRSA infections, is less effective than oxacillin for treating infections caused by methicillin-resistant S. aureus (MSSA) (6, 40, 49) and therefore cannot be used to treat all S. aureus infections. In addition, the indiscriminate use of glycopeptides may facilitate the emergence of S. aureus strains with decreased susceptibility or resistance (35).

Two questions, therefore, arise when clusters of gram-positive cocci, suggesting the presence of staphylococci, are first detected on a smear from a positive BC bottle: (i) is the organism S. aureus or CoNS, and (ii) is it susceptible to methicillin? Conventional bacteriologic methods require 24 to 48 h to answer these questions. Several faster methods of differentiating between S. aureus and CoNS in BC bottles in 150 to 250 min have been assessed (7), but their results do not indicate whether or not the strain is susceptible to methicillin. Direct real-time PCR (RT-PCR) in BC-positive bottles has been used to detect the mecA gene encoding PBP2a, which causes methicillin resistance, but as stated above, it does not differentiate CoNS from S. aureus (8). A duplex RT-PCR detecting the mecA gene and Sa442 (36) or the nuc (43) or femA gene (1) specific for S. aureus has been proposed to do it, but its efficacy has not been evaluated when used with the different commercially available BC bottles, some of which include potential PCR inhibitors like resins (1, 36) or charcoal particles (43). In our study, we used a triplex RT-PCR, which avoids these problems by targeting femA and mecA genes as well as an rrS sequence specific for staphylococci. We assessed its efficacy.
with the main types of BC bottles currently used by most microbiology laboratories and evaluated its potential ability to improve the effectiveness of antibiotic treatment.

**MATERIALS AND METHODS**

The study was undertaken in four French teaching hospitals located in the Paris, France, area (1,100, 670, 580, and 540 beds, respectively). BC bottles from two of these hospitals were processed using the Bactec 9240 system (Becton-Dickinson, Le Pont-De-Claix, France), those from another using the BacT/Alert 3D system (bioMérieux, Marcy l’Etoile, France) with charcoal, and those from the remaining hospital using the latter system but without charcoal.

The types of BC bottles used were Lytic/10 Anaerobic/F and Plus + Aerobic/F bottles (Becton-Dickinson). All patients over 18 years of age whose BCs were positive for clustered gram-positive cocci on direct smear examination (DSE) between 1 March and 31 August 2004 were included in the study. When a patient had more than one positive BC, only the first was included.

One milliliter of the contents of each bottle included was stored at 20°C, and the bottle was then processed using standard bacteriologic techniques. Differentiation between *S. aureus* and CoNS was based on the results of the rabbit plasma coagulate test (bioMérieux) and SlideX Staph Plus kit (bioMérieux), and the results were interpreted as described previously (37). Isolate susceptibility to antibiotics, including methicillin, was then determined by the diffusion method (44) and was interpreted as described previously (2). The results of the phenotypic techniques were reported to clinicians according to standard procedures.

One of the investigators visited the patients concerned in their clinical wards and recorded the following information: age, gender, type of infection (community or nosocomial), source of infection, ongoing treatments, therapeutic changes made upon transmission of the phenotypic results, and in-hospital deaths.

The portal of entry was defined as a localized focus of staphylococcal infection preceding bacteremia. Catheter-related staphylococcal bacteremia was defined according to the following criteria: (i) evidence of phlebitis or inflammation at the insertion site and/or (ii) the presence of staphylococci with the same antibiotic susceptibility pattern on semiquantitative catheter tip cultures and no evidence of another source of infection (21). CoNS bacteremia was considered a true BBSI if at least two BCs within 5 days were positive for the same isolate (CoNS isolates of each BC were considered the same when they had identical antibiotic susceptibility patterns) or if one BC was positive and the patient exhibited clinical evidence of infection, including abnormal white blood cell counts, temperature, or blood pressure (3). Staphylococcal bacteremia was classified as nosocomial if the result of the first BC was positive and if clinical evidence of infection developed later than 48 h after admission, or it was classified as community-acquired or of unknown origin when data were omitted.

The efficacy of antibiotic therapy was classified as optimal, suboptimal, or nonoptimal with regard to the antibiotics used and according to the staphylococcal species isolated and its susceptibility to methicillin. For MSSA, treatment with oxacillin or an expanded-spectrum or broad-spectrum cephalosporin or with β-lactam inhibitor combined with a β-lactamase inhibitor was classified as optimal, treatment with glycopeptides (except in cases of allergy to β-lactam) was suboptimal, and treatment with an antibiotic to which the isolate was not susceptible was nonoptimal. For methicillin-resistant staphylococcal BBSI, treatment was classified as optimal when glycopeptides were used and as nonoptimal otherwise.

The frozen aliquots from positive BC bottles were all taken to the same laboratory for analysis by triplex RT-PCR assay. They were defrosted in batches at room temperature, and DNA was directly extracted by a rapid procedure requiring a maximum of 10 min using the DiagHemoc-Staph kit (Diagenode, Liege, Belgium). Briefly, 20 µl of each aliquot was dropped into tubes containing 1 ml of sterile, distilled, deionized water and 50 µl of glass beads. The tubes were incubated for 1 min at room temperature. The suspensions were then centrifuged at 16,000 × g for 1 min, and the pellets were resuspended in 100 µl of lysis buffer and vigorously stirred by vortexing. Nine hundred microliters of RNase/DNase-free water (Sigma-Aldrich, Saint Quentin Fallavier, France) was then added to each tube, and the tubes were spun centrifuged at 16,000 × g.

Triplex RT-PCRs were processed on ABI Prism 7000 sequence detection systems (Applied, Courtaboeuf, France). The primer sequences used for the amplification of the *mecA* gene, the *femA* gene of *S. aureus*, and the signature of staphylococci in the *rrs* gene and the hybridization probes used are given in Table 1. Triplex RT-PCR was performed with a total volume of 25 µl, containing 2.5 µl of master mix PCR buffer, 1 µl of primer and probe mix (Diagenode), and 2 µl of template DNA, prepared as described above. Thermocycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 35 cycles of 15 s at 95°C and 40 s at 60°C. The full thermocycling procedure lasted 84 min. RNase/DNase-free water (Sigma) was used with each batch of samples as a negative control, and positive organisms and purified DNA from MRSA (ATCC 43868) and MSSA (ATCC 25923) were used as positive controls. The cutoff limit of detection, determined for serially purified DNA dilutions, was approximately 100 DNA copies of each target per sample (data not shown).

The study has been approved by our institutional review board.

**RESULTS**

The first BC bottles with clustered gram-positive cocci on DSE from 410 patients (respectively, 111, 59, 73, and 167 from each of the four participating hospitals), were included in the study. The patients comprised 243 males (59%) and 167 females (41%), and their mean age was 58 years (0 to 100). Thirty-four (8%) were hospitalized in surgery wards, 250 (61%) in medical wards, and 126 (31%) in intensive care units (ICU). The results of the standard identification methods showed that 31 BCs (7.6%) were positive for MRSA, 66 (16%) for MSSA, 1 (0.2%) for both MRSA and MSSA, 188 (46%) for methicillin-resistant CoNS (MR-CoNS), 113 (27.5%) for methicillin-susceptible CoNS (MS-CoNS), 4 (1%) for both MR-CoNS and MS-CoNS, and 7 (1.7%) for *Micrococcus* spp.

The results of the triplex RT-PCR compared to those for the phenotypic species identification and methicillin susceptibility determination methods are shown in Table 2. The *femA* gene was detected in all the BCs which contained *S. aureus* (n = 98) but in none of those which did not (n = 312). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the triplex RT-PCR for the detection of *S. aureus* were therefore all 100%. The *mecA* gene was detected in 31 BCs containing MRSA, 1 BC containing a

**TABLE 1. Primers and fluorogenic probes used for triplex RT-PCR**

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Forward and reverse primer sequences</th>
<th>Fluorogenic probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>meca</td>
<td>5'-GAAAATTTAAAAATCAGAAC GTGTTAA-3'</td>
<td>5'-GACCGAAACAATGTGGGA TTG6C3A-3'</td>
</tr>
<tr>
<td>femA</td>
<td>5'-TGCTGGTTGTTACATCAA-3'</td>
<td>5'-ATTITGGCGGAAGATGATG ACGTCAAAATG3-3'</td>
</tr>
<tr>
<td>Signature of staphylococci in the <em>rrs</em> gene</td>
<td>5'-ACCTGCTCCCTGGCTAGG3-3'</td>
<td>5'-CCACACTGGAAACTGAGAC ACGTCC-3'</td>
</tr>
</tbody>
</table>
TABLE 2. Comparison of the results of triplex RT-PCR and phenotypic identification with the results for methicillin susceptibility tests for the various types of bacteria present in bottles containing BCs from 410 patients with gram-positive cocci on DSE

<table>
<thead>
<tr>
<th>Bacteria detected by phenotypic methods (no. of positive BCs)</th>
<th>No. of BCs positive for the indicated bacteria detected by RT-PCR*</th>
<th>Species other than staphylococci</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSSA (66)</td>
<td>66 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>MRSA (31)</td>
<td>0 31 0 0</td>
<td>0</td>
</tr>
<tr>
<td>MS-CoNS (113)</td>
<td>0 0 109 4</td>
<td>0</td>
</tr>
<tr>
<td>MR-CoNS (188)</td>
<td>0 0 0 188</td>
<td>0</td>
</tr>
<tr>
<td>Micrococcus spp. (7)</td>
<td>0 0 0 0</td>
<td>7</td>
</tr>
<tr>
<td>MRSA + MSSA (1)</td>
<td>0 1 0 0</td>
<td>0</td>
</tr>
<tr>
<td>MR-CoNS + MS-CoNS (4)</td>
<td>0 0 0 4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Determined by the amplification of mecA, femA, and the rs signature of staphylococci, respectively, as follows: negative, positive, and positive for MSSA; positive, positive, and positive for MRSA; negative, negative, and positive for MS-CoNS; positive, negative, and positive for MR-CoNS; and negative, negative, and negative for species other than staphylococci.

mixture of MRSA and MSSA, 188 BCs containing MR-CoNS, 4 BCs containing a mixture of MR-CoNS and MS-CoNS, and 4 BCs in which the only species isolated by the phenotypic techniques was MS-CoNS. However, by PCR performed with DNA extracted from the colonies, these four isolates were positive for mecA and were therefore classified as MR-CoNS and not MS-CoNS. Consequently, the sensitivity and PPV of the triplex RT-PCR for the detection of MR staphylococci were 100% and the specificity and NPV were greater than 100% compared to those of the phenotypic techniques. The rs signature for staphylococci was detected in all the BCs containing staphylococci ($n = 403$) but in none containing Micrococcus spp. Therefore, the sensitivity, specificity, PPV, and NPV of the triplex RT-PCR for the detection of staphylococci were all 100%.

Clinical characteristics were not recorded for 37 patients. They comprised 13 (2 with MRSA, 7 with MR-CoNS, and 4 with MS-CoNS) who died within 24 h before BC positivity was detected, 2 (1 with MSSA and 1 with MS-CoNS) who refused to be hospitalized, 6 (2 with MSSA, 3 with MS-CoNS, and 1 with Micrococcus spp.) who were not hospitalized, and 16 (1 with MRSA, 6 with MSSA, 6 with MR-CoNS, and 3 with MS-CoNS) for whom clinical information could not be obtained. For 252 of the remaining 373 patients, BC results were judged to be devoid of clinical significance (see definitions above). The 252 included 246 with CoNS and 6 with Micrococcus spp. For three of the latter group, treatment had in fact been started when the DSE results became known but was stopped for all three when the bacterial identification was completed. The BC results were judged clinically significant for 121 patients, including all of the 86 with S. aureus (57 with MSSA and 29 with MRSA) and the 35 with CoNS (17 with MS-CoNS and 18 with MR-CoNS). Sixty-eight (56%) of the 121 patients with true BBSI had already been treated with antibiotics when their BCs were diagnosed positive (Fig. 1A). This treatment was retrospec-

vatively judged nonoptimal in 19 (28%), suboptimal in 10 (15%), and optimal in 39 (57%) (Fig. 1A). After the transmission of the DSE results to clinicians, several changes occurred. First, 13 of the 19 nonoptimal treatments were changed to optimal ones and 1 to a suboptimal one, and 5 remained nonoptimal. After the full species and methicillin susceptibility were determined 24 to 48 h later by standard techniques, further changes were observed in this group of patients. In the end, all primitively nonoptimal treatments had ultimately been changed to optimal ones. Second, among the 10 patients whose initial treatments were suboptimal, 2 were switched to optimal ones after the DSE results were available, and a total of 6 were so treated after all phenotypic results were available. Four remained suboptimally treated. Last, changes in the 39 patients whose treatments were initially optimal resulted in switches to suboptimal ones for 9 after their DSE results were known, but for all of the 9, the treatment was indeed readjusted to an optimal treatment after their full phenotypic results were known. The details on the bacterial species isolated in each category of patients are shown in Fig. 1A.

Disclosure of the DSE results also induced major changes in the care of 53 (44%) of the 121 true BBSI patients who had not been treated until then (Fig. 1B). Optimal treatments were initiated in 31 (58%) of them, while 14 received suboptimal treatments and 1 nonoptimal treatment; meanwhile, 7 patients remained untreated. After the full phenotypic results were known, the number of patients treated optimally increased to 51, the last 2 remaining suboptimally treated (see Fig. 1B for the details on the bacterial species isolated in each patient category).

Altogether, when considering all of the 121 patients with true BBSI (Fig. 1A and B), 76 (63%; 29 with MSSA, 24 with MRSA, 6 with MS-CoNS, and 17 with MR-CoNS) received optimal treatment after the DSE results were known, and the number climbed to 115 (95%; 55 with MSSA, 29 with MRSA, 13 with MS-CoNS, and 18 with MR-CoNS) when all of the phenotypic results became available 24 to 48 h later. No patient remained untreated, but six (two with MSSA and four with MS-CoNS) were still treated suboptimally then.

We evaluated the potential impact of the faster triplex RT-PCR results by comparing the ideal treatments that could have been initiated immediately after obtaining the DSE results if the triplex RT-PCR results had been available to the treatments and 1 nonoptimal treatment; meanwhile, 7 patients remained untreated. After the full phenotypic results were known, the number of patients treated optimally increased to 51, the last 2 remaining suboptimally treated (see Fig. 1B for the details on the bacterial species isolated in each patient category).

First, earlier knowledge of the triplex RT-PCR results could have prevented the overtreatment of three patients without true BBSI who had only one CoNS-positive BC but for whom antibiotics were actually initiated (two with vancomycin and one with oxacillin; results not shown) after disclosure of the results of the DSE and were continued until their phenotype identifications were known 48 h later.

Second, when we analyzed the 121 patients with true BBSI, we observed that for the 68 patients already treated (Fig. 1A) when their BCs were diagnosed positive, the following increases in treatment adequacy could have been made: (i) the 19 with nonoptimal treatment could have all received optimal treatment immediately instead of in a delayed manner, as for 6 of them, (ii) it could have been the
same for at least 4 of the 8 of them with initial suboptimal treatment (for the 4 others, the treatment remained suboptimal even when the full phenotypic results were known), and (iii) the unwise transient changes from optimal to suboptimal treatments observed for 9 of the 39 patients with initial optimal treatment could have been avoided. Also, in the remaining 53 patients with true BBSI who were not already treated (Fig. 1B) when their BCs were diagnosed positive, we observed that treatment adequacy could have been increased for at least 20 of the 22 for which the treatment initiated after the DSE results were known was not optimal (for the 2 others, the treatment remained actually suboptimal even when the full phenotypic results were known).

Thus, in all, 39/121 (32%) patients with true BBSI (26 with MSSA, 5 with MRSA, 7 with MS-CoNS, and 1 with MR-CoNS) could have been treated better using the triplex RT-PCR results. The treatments that they actually received are presented in FIG. 1. Characteristics of antibiotic treatments for the 68 patients already treated (A) and for the 53 untreated (B) when a first BC bottle containing clustered gram-positive cocci was detected by DSE. From the top down, the three zones in each figure, respectively, refer to the treatments actually received before the detection of BC positivity, after the results of the DSE became available, and after disclosure of the complete phenotypic results. pts, patients; Rx, treatment.
Table 3. Characteristics of antibiotic treatments received after disclosure of DSE results for 39 patients with true BBSI who might have benefited from the fast availability of triplex RT-PCR results.

<table>
<thead>
<tr>
<th>Antibiotic treatment</th>
<th>MSSA (n = 26)</th>
<th>MRSA (n = 5)</th>
<th>MS-CoNS (n = 7)</th>
<th>MR-CoNS (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suboptimal Vancomycin</td>
<td>21</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonoptimal Oxacillin</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Strikingly, 26 patients with MS staphylococci (21 with MSSA and 5 with MS-CoNS) received nonoptimal vancomycin.

**DISCUSSION**

Our results showed that triplex RT-PCR would help to make early decisions that would improve the treatment of patients exhibiting a first positive BC with gram-positive cocci in clusters on DSE. RT-PCR results were available in less than 90 min, and the assay was both sensitive and specific, with much higher predictive values than those given by the phenotypic techniques currently used in clinical laboratories. The proper use of triplex RT-PCR results by the clinicians could have helped to make early decisions that would improve the treatment of patients who were initially given nonoptimal antibiotic regimens or who remained untreated although they were in fact infected (the latter group mostly comprised Staphylococcus aureus patients) and, second, because it could have helped clinicians to refrain from treating the patients not actually suffering from an infection, most of whom had BCs contaminated by CoNS but were not in fact infected.

Of the 86 patients from our study with true S. aureus BBSI, 10 (11%) had still not been adequately treated 24 to 48 h after the DSE results were known (5 patients were untreated, and 5 had been given nonoptimal treatments). It is known that for ICU patients with S. aureus BBSI, treatment inadequacy can reach 30% (16, 47), a figure that might rise even higher with the spread of the current epidemic of community-acquired MRSA infections (26). Because delayed treatment can increase the rates of prolonged hospitalization (22) and of secondary metastatic infections, such as endocarditis (10, 34) and vertebral osteomyelitis (17), the use of triplex RT-PCR should reduce both of these unwholesome events by optimizing early treatments. Another retrospective study has also shown that there was a high percentage of patients nonoptimally treated
The value of the PCR has also been shown in a prospective, however small, study (13). We found that only 35 (12%) of 121 patients with CoNS-positive BCs were actually infected and that 8 (23%) of them were not initially given optimal treatment (2 were untreated, 1 was given a nonoptimal treatment, and 5 a suboptimal treatment). These patients might also have benefited from the early results of our assay, although the impact of delayed treatment on patients infected by CoNS has never, to our knowledge, been quantified. Our result is in agreement with those reported by others (13).

The 21 (36.8%) patients of 57 with MSSA who received suboptimal vancomycin and were switched to optimal treatments only 24 to 48 h after the results of the DSE were known would also have benefited from earlier results. This result is in agreement with those of other studies (13, 42). The early use of optimal β-lactams, especially oxacillin, is important because they are more rapidly bactericidal than glycopeptides on staphylococci in vitro (25), resulting in better clinical efficacy in patients with MSSA endocarditis (6) and in hemodialyzed patients suffering from MSSA bacteremia (40).

The same might be true of the five patients given vancomycin who were infected with MS-CoNS. Our result is in agreement with those described by others (13). However, for these patients, the benefit from early triplex RT-PCR results is more hypothetical because no data proving that early oxacillin is clinically beneficial for MS-CoNS patients are available.

The last group of patients who might have benefited from the early results of the triplex RT-PCR were the three with BC-positive CoNS who were not actually infected but were given antibiotics unnecessarily for 24 to 48 h before phenotypic results were known. The same observation was also reported elsewhere (42). For these patients, the risks of allergy and unjustified selective pressure could have been avoided.

In all, triplex RT-PCR could have prevented the treatment of 28 patients (including 26 [21%] of the 121 with true BBSI and 2 [8%] of the 252 with false BBBSI) with glycopeptides and the associated risk of the emergence of glycopeptide resistance in enterococci (27, 32), as well as the risk of either decreased susceptibility to glycopeptides or glycopeptide resistance in staphylococci (5). Obviously, the clinical usefulness of this test may depend on the patient case mix and local rates of appropriate empirical therapy for septic patients. In summary, the clinical impact of a PCR assay for identification of bacteremia caused by Staphylococcus aureus and determination of methicillin resistance directly from blood cultures. J. Clin. Microbiol. 41:1394–1394.


41. Reference deleted.


