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We evaluated the Hyplex BloodScreen PCR–enzyme-linked immunosorbent assay (ELISA) system (BAG, Lich, Germany), a new diagnostic test for the direct identification of gram-negative bacilli and gram-positive cocci from positive blood cultures, with 482 positive BACTEC 9240 blood culture bottles. The test involves amplification of the bacterial DNA by multiplex PCR and subsequent hybridization of the PCR products to specific oligonucleotide probes in an ELISA-based format. The available probes allow the separate detection of Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes, Klebsiella spp., Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis/Enterococcus faecium, Streptococcus pyogenes, and Streptococcus pneumoniae and the staphylococcal mecA gene. The Hyplex BloodScreen test showed an overall sensitivity of 100% for the identification of gram-negative bacilli and 96.6 to 100% for the identification of gram-positive cocci (S. aureus, 100%; S. epidermidis, 97.2%; Enterococcus faecalis/Enterococcus faecium, 96.6%; and Streptococcus pneumoniae, 100%). The specificities of the test modules ranged from 92.5 to 100% for gram-negative bacilli and 97.7 to 100% for gram-positive cocci (Escherichia coli, 92.5%; Pseudomonas aeruginosa, 98.5%; Klebsiella spp., 100%; Enterobacter aerogenes, 100%; S. aureus, 100%; S. epidermidis, 97.7%; Enterococcus faecalis/Enterococcus faecium, 99.6%; Streptococcus pyogenes, 100%; and Streptococcus pneumoniae, 99.3%). The result of the mecA gene detection module correlates with the result of the phenotypic oxacillin resistance testing in all 38 isolates of Staphylococcus aureus investigated. In conclusion, the Hyplex BloodScreen PCR-ELISA system is well suited for the direct and specific identification of the most common pathogenic bacteria and the direct detection of the mecA gene of Staphylococcus aureus in positive blood cultures.

Bloodstream infections are potentially life-threatening conditions that require rapid identification of the causative agent in order to facilitate a specific antimicrobial therapy. Rapid bacterial identification and susceptibility testing not only improves patient therapy and outcome but can also reduce costs and may prevent the development of bacterial resistance, for instance by allowing a shorter duration of antimicrobial therapy or an early switch from empirically administered broad-spectrum antibiotics to narrow-spectrum substances (8, 15, 18).

Several methods for direct detection and susceptibility testing of bacteria in positive blood cultures have been described, including PCR methods, as well as DNA and RNA probes and restriction fragment length polymorphism profile analysis (4). The target sequences of the primers and probes were the eubacterial 16S rRNA gene and family-, genus-, or species-specific genes for identification of the bacteria, as well as specific resistance genes, such as the staphylococcal mecA gene, for the determination of antimicrobial susceptibility (2, 5, 9–12). The application of fluorescence-based real-time PCR even allowed specific detection of pathogenic bacteria in blood cultures within a few hours (17). However, there is no commercial kit for molecular diagnostics of blood cultures yet available and standardization of the assays is lacking. In addition to molecular methods, the Vitek 2 system has recently been evaluated for the identification and susceptibility testing of pathogenic bacteria by direct inoculation from positive BACTEC blood culture bottles and showed promising results (13). However, thus far these evaluations have exclusively used gram-negative bacilli, and problems with the identification of gram-positive cocci have been described (1).

Recently, a commercially available test kit, the Hyplex BloodScreen multiplex PCR–enzyme-linked immunosorbent assay (ELISA) system (BAG, Lich, Germany), has been developed that facilitates direct identification of pathogenic bacteria from positive blood culture bottles within few hours. Independently of the result of the initial Gram stain, a PCR assay for either gram-positive or gram-negative bacteria is applied. For subsequent hybridization in an ELISA-based format, the assay is made up of a panel of test modules (microtiter plate cavities coated with a specific probe) for the identification of Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes, Klebsiella spp., Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, and Enterococcus faecalis/Enterococcus faecium. In addition, the staphylococcal mecA gene can be detected with a specific test.

In the present study, we first evaluated the Hyplex BloodScreen multiplex PCR-ELISA system for the direct identification of pathogenic bacteria and the detection of the staphylo-
cocal meca gene on 482 positive aerobic and anaerobic BACTEC 9240 blood culture bottles. The results of the Hyplex BloodScreen test were compared to the results of culture and biochemical identification as “gold standard.”

MATERIALS AND METHODS

Sample collection and bacterial strains. Samples consisted of 482 positive BACTEC 9240 blood culture bottles, including 376 aerobic and 106 anaerobic bottles from patients of all departments of the hospital of Ulm University. Samples were collected between July 2001 and August 2002 after automatic detection of the bottles in the BACTEC 9240 (Becton Dickinson) blood culture system and processed in parallel to routine diagnostics. On each day of collection, all blood cultures positive with gram-positive cocci, gram-negative cocci, or gram-negative rods in the initial Gram stain, including mixtures of different bacteria, were used, and no preselection of samples was done. In cases where both the aerobic and the anaerobic bottle were detected to be positive, only the aerobic bottle was included in the study.

Cultural identification of bacteria. Identification and differentiation of bacteria grown in BACTEC 9240 bottles was performed according to the results of the Gram stain. Identification of all bacteria apart from most staphylococci was done by Api (Api 20 Strep, Api Rapid ID 32 Strep, Api NH, Api 20E, and Api 20NE [all from BioMerieux]). For staphylococcal diagnosis, identification was based on typical morphological (color, hemolysis, etc.), coagulase, and detection by hybridization. Positive samples are identified and differentiation was ambiguous, an Api Staph analysis was performed.

Susceptibility testing. Methicillin resistance in S. aureus was detected by determination of PBP2a by a latex agglutination test (MRSA Screen; Innogenetics, Ghent, Belgium) and phenotypically by growth on Mueller-Hinton agar supplemented with 6 ug of oxacillin and 4% NaCl (Heipha)/ml. In coagulase-negative staphylococci (CNS), oxacillin resistance was determined phenotypically by agar diffusion test according to the NCCLS guideline M100-S11, with a 1-ug oxacillin disk on Muller-Hinton agar supplemented with 2% NaCl. An inhibition zone of <17 mm indicated resistance to oxacillin. In addition, oxacillin MIC was determined by microbroth (broth) dilution on the Merlin Micronaut System (Merlin) by using a range of 0.25 to 32 ug of oxacillin of 2% NaCl in the assay. In all strains, the results of both methods were consistent.

DNA preparation. Total DNA from positive blood culture bottles was prepared by an alkaline lysis method, according to the protocol of Millar et al. (14). Briefly, 0.5 ml of inoculated blood culture medium was mixed with 1.0 ml of alkali wash solution (0.5 M NaOH, 0.05 M sodium citrate) and then further mixed on a rotator for 10 min at room temperature. The suspension was centrifuged at 13,000 × g for 5 min, the pellet was washed twice in 0.5 ml of 0.5 M Tris-HCl (pH 8.0) and centrifuged as described above, and the resulting pellet was resuspended in 0.1 ml of Tris-EDTA (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The suspension was transferred in a screw-cap reaction tube, incubated at 95°C for 10 min, and subjected to two cycles of 2 min of freezing in liquid nitrogen and 2 min of boiling in a boiling water bath (freezing at −20°C for 5 to 10 min each was also effective). After a centrifugation step at 13,000 × g for 15 min, the supernatant was stored at −20°C until use in the Hyplex BloodScreen test.

Hyplex BloodScreen multiplex PCR-ELISA system. The Hyplex BloodScreen multiplex PCR-ELISA system (version 1; BAG) involves an initial amplification of the bacterial DNA by multiplex PCR and a subsequent hybridization of the PCR product to specific oligonucleotide probes in an ELISA-based format with color-coded wells. The test takes ca. 4.5 to 6 h, including DNA isolation, PCR amplification, and detection by hybridization. Positive samples are identified by measurement of the optical densities (ODs) in the ELISA plate wells. An OD of <0.2 has been defined as negative, an OD between 0.2 and 0.4 has been defined as borderline, and an OD of >0.4 has been defined as positive by the manufacturer. All samples with borderline results (<2% of all samples) were repeated three times (extending the turnaround time of the assay for ~2.5 h), and the means of the measurements were considered. The system includes a gram-positive PCR kit for the amplification of the DNA of gram-positive bacteria and test modules (microtiter plate cavities coated with a specific probe) for the detection of S. aureus, S. epidermidis, E. faecalis/E. faecium, S. pyogenes, S. pneumoniae, and the staphylococcal meca gene, as well as a gram-negative PCR kit for the amplification of the DNA of gram-negative bacteria and test modules for the detection of E. coli, P. aeruginosa, E. aerogenes, and Klebsiella spp. Target genes of the assay are species-specific housekeeping genes. The test was performed with samples evaluated by the manufacturer except for the use of 0.5-ml tubes (instead of 0.2-ml tubes) for the multiplex PCR on a thermal cycler (Thermal Cycler Touch Down; Hybaid, Ashford, United Kingdom) and the following modification of the PCR protocol: a denaturation time of 1 min, an annealing time of 1 min, and an elongation time of 90 s.

Nucleotide sequence analysis of PCR products. The nucleotide sequences of ampiclons were determined in eight samples in which the biochemical identification of certain bacterial isolates was found to be ambiguous or divergent with respect to the PCR result. Briefly, the primers 16sfor (AGAGTTTGATCTGGCTCACG) and 16srev (GTTTATCTGTAGACTCGT) were used as sequencing primers; these primers span a region from positions 8 to 150 of the eubacterial 16S rRNA gene (7). Amplification products were purified by using the HighPure PCR product purification kit (Roche Diagnostics), and cycle sequencing reactions of the 16S rDNA sequence were performed as described in the dye terminator cycle sequencing ready reaction kit (ABI Prism Biosystems). The fluorescence-labeled reaction products were analyzed with an ABI Prism 310 genetic analyzer. Obtained sequences were compared to the GenBank/EMBL database for identification of isolates. Identification was defined as a sequence homology of ≥98.5% with the respective strain in the database.

LightCycler PCR assays. For confirmation of the identification of S. epidermidis and the meca gene in selected samples, LightCycler PCR assays with hybridization probes were performed. For the identification of S. epidermidis, primer DG74 and RW01, amplifying a 386-bp fragment of the 16S rRNA gene, and the probes EptD and EptLC were used according to the protocol published by Wellin resign et al. (17). For identification of the meca gene, primers Mec-F2 and Mec-R2 and probes Mec-HP-1 and Mec-HP-2 were used strictly according to the protocol published by Reischl et al. (16).

RESULTS

Identification of samples with gram-negative microscopy. In 140 blood cultures, exclusively gram-negative bacteria were visible in the initial Gram stain and grown in pure culture. In all samples, the PCR assay for gram-negative bacteria and the hybridization modules for E. coli, P. aeruginosa, Klebsiella spp., and E. aerogenes were applied. Compared to culture and subsequent biochemical identification, the diagnostic sensitivity of the assays resulted in 100% (Table 1). For the E. aerogenes assay, the sensitivity was not determined since no isolate of this species was included in the panel of samples tested. Regarding diagnostic specificity, the Klebsiella spp. and the E. aerogenes assay also had a specificity of 100%. The P. aeruginosa assay showed a specificity of 98.5% since cross-reactions with Citrobacter koseri and Enterobacter cloacae were observed. However, these false-positive results had much lower OD values than the true positive isolates and only reached the cutoff OD value of 0.4, which discriminates borderline and positive samples, as stated by the manufacturer (Table 1). The E. coli assay showed a specificity of only 92.5% since cross-reactions with Bacteroides fragilis, E. cloacae, and Morganella morganii were observed. Although OD values of the false-positive samples that contained B. fragilis and M. morganii clearly differed from the OD values from the true positive samples (median OD of <0.6 versus 2.5, P < 0.001 by Mann-Whitney U test; see also Table 1), the false-positive E. cloacae isolate had an OD value similar to that of E. coli (Table 1). Identification of this E. cloacae isolate was further confirmed by 16S rDNA sequencing (99.6% homology to the respective strain in the database).

Identification of samples with gram-positive microscopy. In 309 blood cultures, exclusively gram-positive cocci were visible in the initial Gram stain and grew in pure culture. In all samples, the PCR assay for gram-positive bacteria and the hybridization modules for S. aureus, S. epidermidis, E. faecalis/ E. faecium/ E. faecalis, S. pyogenes, and S. pneumoniae, as well as the meca gene, were applied. A sensitivity of 100% was observed for the S. aureus and S. pneumoniae modules. The E. faecalis/E. fæ-
The diagnostic sensitivities and specificities for E. coli were 100 and 98.5%, 100 and 100%, and not determined and 100%, respectively. True-positive results are in boldface.

Identical results were obtained in eight of the nine samples using the LightCycler PCR. The remaining sample, which was positive for S. pyogenes in the Hyplex BloodScreen test but negative in the LightCycler PCR, was confirmed to be negative in the S. pyogenes LightCycler PCR. Concerning strains with mecA-negative results, the mecA PCR was performed in all samples showing gram-positive bacteria. The mecA gene LightCycler PCR was performed. This PCR confirmed the presence of the mecA gene in all false-negative isolates. Both “false-positive” isolates were also confirmed to carry the mecA gene and therefore have to be considered as true positives.

Concerning CNS except S. epidermidis, the Hyplex BloodScreen mecA test yielded false-negative results in 12 of 20 phenotypically resistant isolates, and the additionally performed mecA gene LightCycler PCR confirmed the presence of the mecA gene in all isolates. Interestingly, 9 of 12 isolates were identified as S. haemolyticus. All phenotypically susceptible CNS were correctly identified by the mecA test (Table 3).

Characterization of samples showing a mixture of gram-negative and gram-positive bacteria. A mixed culture of different species of bacteria was grown in 33 samples. In 22 samples a
In the present study, the Hyplex BloodScreen PCR-ELISA system was evaluated for the first time for direct identification of pathogenic bacteria in a large panel of positive BACTEC 9240 blood culture bottles. This test system allows identification of the bacteria within ca. 4.5 to 6 h, including DNA isolation, PCR amplification, and detection by reverse hybridization. Therefore, in contrast to conventional culture and biochemical identification techniques, which usually take 1 to 2 days, the Hyplex BloodScreen PCR-ELISA system generates results much quicker.

Concerning pure cultures of bacteria, the Hyplex BloodScreen PCR-ELISA system allows identification of S. epidermidis in four samples containing mixtures of different gram-positive cocci. Interestingly, in all four samples the additionally performed S. epidermidis PCR was negative, suggesting either low amounts of DNA in the sample or contamination of the culture plates. In four samples, bacterial species were identified by the Hyplex BloodScreen test that were not grown in culture, including Klebsiella spp. in two samples and both S. aureus and S. epidermidis in another two samples (Table 4). Since the S. epidermidis PCR was also positive in both samples growing S. epidermidis, the Hyplex BloodScreen test result might be regarded as true positive. The additional detection of Klebsiella spp. in two samples appears plausible since both samples were obtained from the same patient treated in the MICU for sepsis and large-bowel necrosis. The two samples positive for S. aureus were from two patients with abdominal sepsis and urosepsis, respectively.

The remaining sample with culture positive for S. aureus was negative, suggesting either low amounts of DNA in the sample. For E. faecium was repeatedly tested with borderline results (OD = 0.23), most probably due to low amount of DNA in the sample. Concerning pure cultures of bacteria, the Hyplex BloodScreen PCR-ELISA system allows identification of S. epidermidis in four samples containing mixtures of different gram-positive cocci. Interestingly, in all four samples the additionally performed S. epidermidis PCR was negative, suggesting either low amounts of DNA in the sample or contamination of the culture plates. In four samples, bacterial species were identified by the Hyplex BloodScreen test that were not grown in culture, including Klebsiella spp. in two samples and both S. aureus and S. epidermidis in another two samples (Table 4). Since the S. epidermidis PCR was also positive in both samples growing S. epidermidis, the Hyplex BloodScreen test result might be regarded as true positive. The additional detection of Klebsiella spp. in two samples appears plausible since both samples were obtained from the same patient treated in the MICU for sepsis and large-bowel necrosis. The two samples positive for S. aureus were from two patients with abdominal sepsis and urosepsis, respectively.

Of the 15 isolates of S. epidermidis grown in a mixed culture, 13 were phenotypically resistant to oxacillin. In 5 of these 13 isolates meca gene detection was false negative, including all four samples that were false negative for S. epidermidis. False-positive results were not seen with the meca gene detection test.

**DISCUSSION**

In the present study, the Hyplex BloodScreen PCR-ELISA system was evaluated for the first time for direct identification of pathogenic bacteria in a large panel of positive BACTEC 9240 blood culture bottles. This test system allows identification of the bacteria within ca. 4.5 to 6 h, including DNA isolation, PCR amplification, and detection by reverse hybridization. Therefore, in contrast to conventional culture and biochemical identification techniques, which usually take 1 to 2 days, the Hyplex BloodScreen PCR-ELISA system generates results much quicker.

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**TABLE 2. PCR and culture results of blood cultures positive for a single species of gram- positive bacteria**

<table>
<thead>
<tr>
<th>Organism and parameter</th>
<th>No. of strains identified by the Hyplex BloodScreen test</th>
<th>meca gene module</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus Oxacillin resistance (phenotypic) and PRP2a latex test</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Oxacillin susceptibility (phenotypic)</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>S. epidermidis Oxacillin resistance (phenotypic)</td>
<td>142</td>
<td>136</td>
</tr>
<tr>
<td>Oxacillin susceptibility (phenotypic)</td>
<td>36</td>
<td>2*</td>
</tr>
<tr>
<td>Phenotypic susceptibility testing result not available</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*All isolates were positive in the additionally performed meca gene LightCycler PCR.*
The Hyplex BloodScreen PCR-ELISA system had a very high sensitivity, ranging from 96.6 to 100% for the various test modules (Table 1 and 2). The specificities of the different modules were also high and exceeded 97.5% in all assays but one. The test module for the detection of E. coli cross-reacted with B. fragilis, M. morganii, and one isolate of E. cloacae and therefore had a specificity of only 92.5%. Nevertheless, all isolates of Bacteroides spp. were grown exclusively from anaerobic blood culture bottles which should not be applied in the test as stated by the manufacturer.

Interestingly, borderline OD values (OD/H11005 0.2 to 0.4) were exclusively observed in false-positive samples, whereas most (336 of 336) true-positive samples had an OD of >1.0. Therefore, it may be applicable to regard borderline results as negative without a loss in sensitivity. An OD between 0.4 and 0.6 was observed in one true-positive sample, an OD between 0.6 and 0.8 was observed in three samples, and an OD between 0.8 and 1.0 was observed in two samples. Altogether, the Hyplex BloodScreen PCR-ELISA system detected 74.8% (336 of 449) of all bacteria, i.e., 61.4% of gram-negative bacilli and 80.9% of gram-positive cocci, directly in the positive blood culture bottle.

Concerning mixed infections with at least two different species of gram-positive and/or gram-negative bacteria, the Hyplex BloodScreen PCR-ELISA system also showed a high sensitivity, identifying all detectable species in 29 of 33 samples. False-positive results compared to the culture results were obtained in 4 of 33 samples.

The Hyplex BloodScreen PCR-ELISA system not only allows identification of the most important pathogenic bacteria causing bloodstream infections in humans but also includes a test module for the detection of the mecA gene in staphylococci that codes for methicillin susceptibility. In our study, the detection of the mecA gene in S. aureus proved 100% sensitive and specific. Phenotypic detection of methicillin resistance in CNS is difficult due to the heterogeneous expression of the mecA gene in S. aureus.

**TABLE 4. Results of mixed bacterial cultures**

<table>
<thead>
<tr>
<th>Gram stain</th>
<th>Cultured species identifiable by Hyplex BloodScreen test</th>
<th>Cultured species not identifiable by Hyplex BloodScreen test</th>
<th>False-positive results by Hyplex BloodScreen test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-negative rods, gram-positive cocci in chains</td>
<td>P. aeruginosa, E. coli</td>
<td>Acinetobacter spp., P. fluorescens, S. mitis</td>
<td>S. aureus, S. epidermidis</td>
</tr>
<tr>
<td>Gram-negative rods, gram-positive diplococci</td>
<td>E. coli</td>
<td>S. oralis, P. mirabilis, S. mitis</td>
<td></td>
</tr>
<tr>
<td>Gram-negative rods, gram-positive cocci in clusters</td>
<td>E. coli, S. epidermidis</td>
<td>P. aeruginosa, S. epidermidis</td>
<td></td>
</tr>
<tr>
<td>Gram-positive cocci in chains</td>
<td>E. faecalis, E. faecium</td>
<td>S. mitis, S. salivarius</td>
<td></td>
</tr>
<tr>
<td>Gram-positive cocci in clusters</td>
<td>E. faecalis, S. epidermidis</td>
<td>S. mitis</td>
<td></td>
</tr>
<tr>
<td>Gram-positive cocci in chains and in clusters</td>
<td>E. faecalis</td>
<td>S. epidermidis</td>
<td></td>
</tr>
</tbody>
</table>

Species printed in boldface were identified with the specific module of the Hyplex BloodScreen test. S. epidermidis-specific LightCycler PCR also positive. S. epidermidis-specific LightCycler PCR negative.
phenotypic oxacillin susceptibility in CNS was high. The only a negative Hyplex BloodScreen
methicillin resistance (3, 6, 19). In our study, the correlation of reported to be the gold standard for the determination of S. haemolyticus
LightCycler PCR. However, in firmed to harbor the detection in phenotypically oxacillin-resistant isolates was lower (95.8% in pure cultures of S. epidermidis, and only 40.0% in CNS other than S. epidermidis). Thus, the results of the mecA gene PCR of the Hyplex BloodScreen system must be interpreted carefully in the absence of S. aureus and S. epidermidis.

Due to the minimal technical prerequisites that are needed by the test, including only a thermal cycler, an incubator for the hybridization, and a standard ELISA reader or an automated ELISA processor, the test is suited for both large laboratories and smaller laboratory units, e.g., in teaching or district hospitals. The costs of the kit and reagents are moderate and amount to ca. $4.50 (U.S. dollars) per test.

Although it is stated by the manufacturer that the test should only be used on aerobic blood culture bottles, we decided, since all of the species covered by the test represent aerobically growing bacteria, to include both aerobic and anaerobic blood culture bottles since, in our experience, aerobically growing bacteria, especially staphylococci and Enterobacteriaceae, are sometimes found in aerobic blood culture bottles. For instance, in our study 29 of 133 aerobic or facultative anaerobic gram-negative rods, including 16 of 58 isolates of E. coli, were detected in the anaerobic blood culture bottle.

In conclusion, the Hyplex BloodScreen PCR-ELISA system is well suited for the direct and specific identification of the most common pathogenic bacteria in positive blood cultures. It allows earlier identification of pathogenic bacteria compared to routine cultures and may contribute to a timely and cost-effective pathogen-adapted antimicrobial therapy even before availability of phenotypic antimicrobial susceptibility testing results. In addition, it also allows early sensitive and specific detection of the mecA gene in S. aureus.

REFERENCES