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Comparison of direct disk diffusion and standard microtitre broth dilution susceptibility testing of blood culture isolates

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Bloodstream infections are life-threatening conditions which require timely initiation of appropriate antimicrobial therapy. The accuracy of direct disk diffusion susceptibility testing of positive blood cultures was investigated, including for the first time β-lactam/β-lactam-inhibitor combination antibiotics. Results of direct testing, following the guidelines of the Clinical and Laboratory Standards Institute, were compared to standard microtitre broth dilution susceptibility testing of the subcultured isolate on the Merlin MICRONAUT system. Altogether, 758 isolates and 4930 organism/antibiotic combinations from 590 patients were evaluated. With regard to Gram-positive cocci ($n=532$), agreement between both methods was found in 93.9 % of cases, with 1.6 % very major, 1.1 % major and 2.6 % minor errors. For Gram-negative rods ($n=226$), agreement was found in 91.9 % of cases, with 1.2 % very major, 0.7 % major and 6.3 % minor errors. When applying the breakpoints of the Deutsches Institut für Normung for interpretation of MICRONAUT tests, agreement of direct disk diffusion with standard testing decreased to 82.4 % in Gram-negative rods, with 3.6 % very major, 0.5 % major and 13.4 % minor errors. A high rate of disagreement was observed with oxacillin and gentamicin in Gram-positive cocci, and with cefuroxime, amoxycillin/clavulanate and piperacillin/tazobactam in Gram-negative rods. In conclusion, the limitations of direct disk diffusion testing of positive blood cultures must be kept in mind by the clinical microbiologist and should, where necessary, be communicated to the clinician to ensure adequate treatment of severely ill patients.

INTRODUCTION

Bloodstream infections are life-threatening conditions which require rapid initiation of specific antimicrobial therapy. Adequate first-line antimicrobial therapy has a positive impact on the outcome of bacteraemic patients (Harbarth *et al.*, 2003; Kollef *et al.*, 1999). In addition, early targeted antimicrobial therapy can also reduce costs and may prevent development of bacterial resistance (Barenfanger *et al.*, 1999; Doern *et al.*, 1994). Direct antimicrobial susceptibility testing (DAST) of bacterial isolates from positive blood cultures is a well established and recommended method in the diagnostic work-up of bloodstream infections (Baron *et al.*, 2005; Seifert *et al.*, 1997) since it enables the clinician to commence an effective antimicrobial therapy or to change an inappropriate regimen.

DAST of positive blood cultures is usually done by the disk diffusion method, which has the advantages of simplicity and low costs. Early studies from the 1970s and 1980s showed that direct test results gained by the disk diffusion or agar dilution methods correlated acceptably with the results of standard methods (Coyle *et al.*, 1984; Doern *et al.*, 1981; Johnson & Washington, 1976; Mirrett, 1994; Mirrett & Reller, 1979; Wegner *et al.*, 1976). Today, standard antimicrobial susceptibility testing of bacterial isolates obtained from blood cultures is mostly done by microtitre broth dilution techniques using automated systems, such as MICRONAUT (Merlin Diagnostika), Microscan (DADE Behring), Phoenix (BD) or Vitek (BioMérieux).

We recognized in our laboratory that direct disk diffusion test results, especially for β-lactam antibiotics combined with β-lactam inhibitors, such as amoxycillin/clavulanate and piperacillin/tazobactam, from positive blood cultures often differ from the final results obtained by standard microtitre broth dilution. Therefore, we investigated the accuracy of direct disk diffusion susceptibility testing of positive blood cultures by comparing the results of direct disk diffusion of 758 blood culture isolates with the results of standard microtitre broth dilution susceptibility testing using the Merlin MICRONAUT system.

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; DAST, direct antimicrobial susceptibility testing; DIN, Deutsches Institut für Normung.

METHODS

Blood cultures. All positive blood cultures (BACTEC 9240 aerobic, anaerobic and paediatric bottles) with Gram-positive cocci or Gram-negative rods that were processed in our laboratory between 1 January and 31 December 2004 with direct disk diffusion and confirmatory microtitre broth susceptibility testing were included in this retrospective study. For adult patients, both an aerobic and an anaerobic bottle were sent to the laboratory; for paediatric patients, only the paediatric aerobic bottle was sent. An overview of the species distribution found in the blood cultures is given in Table 1. If the same species with an identical antimicrobial susceptibility profile in the standard susceptibility test was isolated several times in one patient within 5 days, only the first isolate was included in the study. Altogether, 758 isolates from 590 patients were included. The isolates were grown from aerobic ($n=128$), anaerobic ($n=135$), paediatric aerobic ($n=80$) or both aerobic and anaerobic bottles ($n=415$). When direct test results from aerobic and anaerobic bottle results were identical, definitive testing was performed on isolates from aerobic subcultures only. Discrepancies between the number of isolates included in the study and the number of direct and standard test results evaluated for each antibiotic were caused by accidental omission of individual antibiotic disks on the agar plates or failure to report the direct test results, failure to analyse individual substances in final testing and different antibiotic disk panels in different bacteria (see below). Blood cultures with polymicrobial growth detected in the Gram stain or in the subcultures were excluded from the study.

Identification of bacterial strains. Identification of all bacterial species, apart from most staphylococci, was done by using API 20 Strep, API Rapid ID 32 Strep, API 20 E and API 20 NE tests (BioMérieux). For staphylococci, diagnosis was based on typical microscopy and morphology (colour, haemolysis, etc.), positive catalase reaction and growth on mannitol/salt agar. *Staphylococcus aureus* was differentiated from coagulase-negative staphylococci by positive clumping factor (Slidex Plus; BioMérieux) and positive aurease detection (BioMérieux). If differentiation was ambiguous, an Api 20 Staph test was performed.

Direct disk diffusion susceptibility testing. Positive blood culture bottles were first analysed by Gram staining. For direct disk diffusion of Gram-positive and Gram-negative isolates, 0.5 and 0.2 ml blood culture medium, respectively, was added to 10 ml 0.9 % sterile saline. This dilution was shown to result in confluent growth on the agar plates used for disk diffusion testing. The suspension was applied to Mueller–Hinton agar plates (Heipha). Oxacillin was tested on Mueller–Hinton agar containing 2 % NaCl. The following panels of antimicrobial disks (BD) were applied: Gram-positive cocci in clusters, doxycyclin (30 μg), erythromycin (15 μg), gentamicin (10 μg), levofloxacin (5 μg), penicillin (10 U), vancomycin (30 μg) and oxacillin (1 μg); Gram-positive cocci in chains and diplococci, ampicillin (10 μg), erythromycin (15 μg), imipenem (10 μg), levofloxacin (5 μg) and vancomycin (30 μg); Gram-negative rods, amoxycillin/clavulanate (20/10 μg), ceftazidime (30 μg), cefuroxime (30 μg), gentamicin (10 μg), imipenem (10 μg), levofloxacin (5 μg) and piperacillin/tazobactam (100/10 μg). Agar plates were incubated at 36 ± 1 °C in ambient air for 18–24 h. Zone inhibition diameters were interpreted according to the standards of the Clinical and Laboratory Standards Institute (CLSI, 2005). Quality control strains, including *Staphylococcus aureus* ATCC 29213, meticillin-resistant *Staphylococcus aureus* ATCC 43300, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853, were investigated daily (each strain three times a week) from pure subcultures and the inhibition zones were within the limits as defined by the CLSI (CLSI, 2005). In addition, direct disk diffusion testing was done with positive blood culture bottles inoculated with the quality control strains in blood from healthy volunteers. Here, the results of the control strains were within the respective limits as well (data not shown).

Standard microtitre broth dilution susceptibility testing. The MICRONAUT system (Merlin Diagnostika) was used for standard susceptibility testing. The MICRONAUT system is an automated

Table 1. Species distribution of the isolates included in this study

Gram-positive species ($n=532$)	Gram-negative species ($n=226$)
Aerococcus viridans (1)	*Acinetobacter* spp. (4)
Enterococcus faecalis (23)	*Citrobacter freundii* (4)
Enterococcus faecium (12)	*Citrobacter* spp. (4)
Enterococcus gallinarum (1)	*Enterobacter cloacae* (7)
Enterococcus spp. (4)	*Enterobacter* spp. (5)
Micrococcus spp. (7)	*Escherichia coli* (110)
Staphylococcus aureus (71)	*Hafnia alvei* (1)
Coagulase-negative staphylococci (413)	*Klebsiella pneumoniae* (22)
	Klebsiella oxytoca (2)
	Morganella morganii (4)
	Pantoea spp. (2)
	Proteus mirabilis (8)
	Pseudomonas aeruginosa (28)
	Pseudomonas spp. (2)
	Rhizobium (*Agrobacterium*) *radiobacter* (1)
	Salmonella Enteritidis (2)
	Serratia marcescens (8)
	Serratia spp. (5)
	Stenotrophomonas maltophilia (7)

microtitre broth dilution susceptibility testing system that uses 394-well microtitre plates. It allows determination of real MICs for up to 25 substances in one plate and bacterial growth in the wells is monitored photometrically. Only pure overnight cultures of the isolates were used for standard MIC determination. Susceptibility testing was performed as recommended by the manufacturer, following the guidelines of the CLSI (2005) concerning inoculum preparation, broth composition and incubation conditions. Merlin MICRONAUT Gram-negative and Gram-positive plates were used for determination of MICs for all substances used in the disk diffusion test as well as a variety of other substances. Interpretation of the MIC values was based on the criteria of the CLSI (2005). In addition, interpretation of the MIC values was done by using the criteria published by the Deutsches Institut für Normung (DIN, 2002) since these criteria are routinely applied in our laboratory. Quality control strains, including the above-mentioned strains as well as the extended spectrum β-lactamase-producing *Klebsiella pneumoniae* ATCC 700603 and an *Enterococcus faecium vanA*-gene-positive strain (Merlin Diagnostika), were investigated daily (each strain three times a week). Since testing of imipenem on the MICRONAUT system is not reliable, standard imipenem testing was done by disk diffusion on pure cultures and, thus, imipenem was not evaluated further in this study.

Data analysis. Agreements and discrepancies in the results of direct disk diffusion and standard microtitre broth susceptibility testing were classified as follows: agreement (identical result in direct and standard testing), very major error (susceptible in direct testing but resistant in standard testing), major error (resistant in direct testing but susceptible in standard testing) and minor error (susceptible or resistant in direct testing and intermediate in standard testing, or vice versa). For comparison of the results, the direct interpretation of the MIC value in standard testing was used to exclude artifacts caused by validation criteria based on results of antibiotics not included in this study.

RESULTS AND DISCUSSION

Study population and inoculum preparation

Direct disk diffusion from positive blood culture and standard microtitre broth dilution testing were performed on 758 organisms and 4930 organism/antibiotic combinations, including 532 Gram-positive and 226 Gram-negative isolates (Table 1). The species spectrum was comparable to that found in other studies and countries (Wisplinghoff *et al.*, 2004).

A critical stage in direct disk diffusion testing is the establishment of the inoculum. As shown by Fay & Oldfather (1979), standardization of the inoculum in DAST from blood cultures is important since different inoculum volumes may cause different inhibition zone diameters (Fay & Oldfather, 1979). They recommended using different inoculum volumes for Gram-positive and Gram-negative organisms. The volumes used in our study were grossly comparable to those recommended by Fay & Oldfather (1979), although our preparation method differs from theirs in dilution of the blood culture medium in sterile saline instead of direct application of the blood culture medium onto the culture plate. In our opinion, inhibitory effects of blood or culture medium components may be diminished by dilution of the positive blood culture medium in sterile saline. With our inoculum preparation method we obtained confluent bacterial growth on the disk diffusion plates. Results of quality control strains artificially spiked into blood culture bottles were within given limits (data not shown).

Data analysis applying CLSI breakpoints

Since DAST is routinely performed in our laboratory following the guidelines of the CLSI (2005), standard microtitre broth dilution test results were at first evaluated following these guidelines as well. Regarding the complete study population, agreement between the results of direct and standard testing was found in 4628 organism/antibiotic combinations, i.e. 93.9 %. Minor errors were seen in 3.6 %, major errors in 1 % and very major errors in 1.5 % of direct disk diffusion test results. Thus, the direct disk diffusion fulfilled the criteria for an antimicrobial susceptibility testing system as proposed by Jorgensen (1993).

For a more detailed view of the results, Gram-positive and Gram-negative bacteria were analysed separately. Gram-positive bacteria were found in 532 of the 758 blood cultures (70.2 %) and were tested in 3587 organism/antibiotic combinations. Agreement of the results was found in 3394 combinations (94.6 %), with minor errors occurring in 2.6 %, major errors in 1.1 % and very major errors in 1.6 % (Table 2). The highest rate of very major errors was found with oxacillin in coagulase-negative staphylococci ($n=21$) and *Micrococcus luteus* ($n=5$), and gentamicin in *Enterococcus faecalis* ($n=1$) and coagulase-negative staphylococci ($n=9$). Very major errors with oxacillin were also reported in a recent study investigating DAST by the Vitek 2 system (Diederen *et al.*, 2006). Unfortunately, the bacterial isolates in our study were not available for further evaluation, such as investigation by *mecA* gene PCR.

Gram-negative bacilli were found in 226 of the 758 blood cultures (29.8 %) and were tested in 1343 organism/antibiotic combinations. Agreement with the test results was seen in 1234 combinations (91.9 %), with minor errors occurring in 6.3 %, major errors in 0.7 % and very major errors in 1.2 % (Table 3). The highest rate of very major errors was found with piperacillin/tazobactam in *Stenotrophomonas maltophilia* ($n=3$), *Pseudomonas aeruginosa* ($n=1$) and *Escherichia coli* ($n=1$), and with cefuroxime in one isolate each of *Pseudomonas fluorescens*, *Escherichia coli*, *Proteus mirabilis* and *Serratia marcescens*. Isolates with very major errors with piperacillin/tazobactam and cefuroxime were different from each other, but one isolate of *Pseudomonas aeruginosa* showed a very major error with ceftazidime and piperacillin/tazobactam and one isolate of *Escherichia coli* had a very major error with amoxycillin/clavulanate and gentamicin. Altogether, the results are comparable to those published with regard to direct and standard antimicrobial susceptibility testing by disk diffusion (Doern *et al.*, 1981, Mirrett & Reller, 1979). Very major errors with second-generation cephalosporins have also been reported in studies that investigated DAST by

Table 2. Correlation of direct disk diffusion and standard antimicrobial susceptibility test results for Gram-positive cocci analysed by applying CLSI breakpoints

Antibiotic	No. (%) of strains with:			
	SIR agreement*	Very major error	Major error	Minor error
Ampicillin	36 (100)	0 (0)	0 (0)	0 (0)
Doxycyclin	457 (93.3)	0 (0)	9 (1.8)	24 (4.9)
Erythromycin	509 (96.4)	3 (0.6)	5 (0.9)	11 (2.1)
Gentamicin	450 (91.6)	10 (2.0)	13 (2.6)	18 (3.7)
Levofloxacin	481 (91.1)	9 (1.7)	3 (0.6)	35 (6.6)
Oxacillin	459 (93.7)	26 (5.3)	1 (0.2)	4 (0.8)
Penicillin	476 (96.4)	9 (1.8)	8 (1.6)	1 (0.2)
Vancomycin	526 (99.2)	2 (0.4)	2 (0.4)	0 (0)
Total (%)	**3394 (94.6)**	**59 (1.6)**	**41 (1.1)**	**93 (2.6)**

*Category agreement with respect to susceptible (S), intermediate (I) and resistant (R) test results.

automated systems (Bruins *et al.*, 2004; de Cueto *et al.*, 2004; Funke & Funke-Kissling, 2004), possibly reflecting decreased stability of these substances in DAST techniques. A high rate of minor errors (6.3 %) was observed in Gram-negative bacilli that were mainly caused by discrepancies with amoxycillin/clavulanate (16.2 % minor errors). Possibly, DAST of β-lactam/β-lactam-inhibitor combination antibiotics is particularly vulnerable to remnants of the patient's blood or components of the blood culture medium that may interfere with the antibiotic.

Data analysis applying DIN breakpoints

Since interpretation of MIC values in the Merlin MICRONAUT system in Germany is usually done by using DIN breakpoints (DIN, 2002), we additionally performed data analysis by using these breakpoints. Agreement between the results of direct and standard testing was found in 4505 organism/antibiotic combinations, i.e. 91.4 %. Minor errors were found in 287 (5.8 %), major errors in 42 (0.9 %) and very major errors in 96 (1.9 %) direct disk diffusion test results.

Regarding Gram-positive cocci, agreement was found in 3398 organism/antibiotic combinations (94.7 %; see Table 4). The highest rate of very major errors was found with oxacillin in coagulase-negative staphylococci ($n=9$), *Micrococcus luteus* ($n=3$) and gentamicin in *Enterococcus faecalis* ($n=1$) and coagulase-negative staphylococci ($n=9$). Thus, results applying the CLSI or DIN breakpoints were comparable apart from a higher percentage of very major errors with oxacillin (5.3 vs 2.4 %) and of major errors with gentamicin (2.6 vs 0.4 %) using the CLSI breakpoints.

For Gram-negative bacilli, agreement was seen in 1107 organism/antibiotic combinations (82.4 %; see Table 5). The highest rate of very major errors was found with cefuroxime in *Escherichia coli* ($n=7$), *Klebsiella pneumoniae* ($n=2$), *Acinetobacter species* ($n=2$), *Serratia marcescens* ($n=2$), *Pseudomonas fluorescens* ($n=1$) and *Proteus mirabilis* ($n=1$), with amoxycillin/clavulanate in *Escherichia coli* ($n=14$) and *Acinetobacter species* ($n=1$), and with piperacillin/tazobactam in *Escherichia coli* ($n=7$), *Stenotrophomonas maltophilia* ($n=3$) and *Pseudomonas aeruginosa* ($n=1$). Very major errors with amoxycillin/clavulanate and

Table 3. Correlation of direct disk diffusion and standard antimicrobial susceptibility test results for Gram-negative bacilli analysed by applying CLSI breakpoints

Antibiotic	No. (%) of strains with:			
	SIR agreement*	Very major error	Major error	Minor error
Amoxycillin/clavulanate	183 (82.4)	1 (0.5)	2 (0.9)	36 (16.2)
Ceftazidime	216 (98.2)	2 (0.9)	1 (0.4)	4 (5.0)
Cefuroxime	200 (88.9)	4 (1.7)	2 (0.9)	19 (8.4)
Gentamicin	216 (96.4)	2 (0.9)	2 (0.9)	4 (1.8)
Levofloxacin	214 (94.7)	2 (0.9)	1 (0.4)	9 (4.0)
Piperacillin/tazobactam	205 (91.9)	5 (2.2)	1 (0.4)	12 (5.3)
Total (%)	**1234 (91.9)**	**16 (1.2)**	**9 (0.7)**	**84 (6.3)**

*Category agreement with respect to susceptible (S), intermediate (I) and resistant (R) test results.

Table 4. Correlation of direct disk diffusion and standard antimicrobial susceptibility test results for Gram-positive cocci analysed by applying DIN breakpoints

Antibiotic	No. (%) of strains with:			
	SIR agreement*	Very major error	Major error	Minor error
Ampicillin	34 (94.4)	0 (0)	0 (0)	2 (5.6)
Doxycyclin	469 (95.7)	2 (0.4)	5 (1.0)	14 (2.8)
Erythromycin	509 (96.4)	4 (0.8)	7 (1.3)	8 (1.5)
Gentamicin	437 (89.0)	10 (2.0)	2 (0.4)	42 (8.6)
Levofloxacin	481 (91.1)	9 (1.7)	3 (0.6)	35 (6.6)
Oxacillin	466 (95.1)	12 (2.4)	8 (1.6)	4 (0.8)
Penicillin	476 (96.4)	9 (1.8)	8 (1.6)	1 (0.2)
Vancomycin	526 (99.2)	1 (0.2)	2 (0.4)	1 (0.2)
Total (%)	**3398 (94.7)**	**47 (1.3)**	**35 (1.0)**	**107 (3.0)**

*Category agreement with respect to susceptible (S), intermediate (I) and resistant (R) test results.

piperacillin/tazobactam were not combined. The above-mentioned very major errors in two isolates of *Pseudomonas aeruginosa* and *Escherichia coli* by applying CLSI breakpoints were also detected by applying DIN breakpoints. Altogether, a much higher rate of very major errors compared to the analysis using the CLSI breakpoints was observed with amoxycillin/clavulanate (6.8 %), cefuroxime (6.7 %) and piperacillin/tazobactam (4.9 %), and in the case of amoxycillin/clavulanate minor errors exceeded 30 % (Table 5).

In conclusion, the high rate of errors with respect to cefuroxime and β-lactam/β-lactam-inhibitor combination antibiotics in Gram-negative bacilli, and oxacillin in Gram-positive cocci considerably restricts the use of the disk diffusion method for DAST of positive blood cultures. These errors may lead to the initiation of inadequate antimicrobial therapy and may have fatal consequences, especially in severely ill patients, where β-lactam/β-lactam-inhibitor combination antibiotics are common first-line substances. In particular, discrepancies have to be considered when different guidelines are followed for DAST and standard repeat testing of blood culture isolates. Therefore, although DAST from positive blood cultures by disk diffusion is an easy-to-perform and cheap method, it cannot be recommended for preliminary testing of the above-mentioned substances.

To overcome the limitations of disk diffusion DAST, implementation of standardized, automated techniques for direct susceptibility testing of blood culture isolates that do not require retesting from subcultures should be targeted. Recent data concerning commercial automated systems, like Vitek (BioMérieux) and Phoenix (BD) (Bruins *et al.*, 2004; de Cueto *et al.*, 2004; Diederen *et al.*, 2006; Funke & Funke-Kissling, 2004; Hansen *et al.*, 2002; Waites *et al.*, 1998), for DAST of blood culture isolates are very promising and, for Gram-negative bacilli, accurate enough to introduce these methods into clinical diagnosis. With regard to DAST of Gram-positive cocci, the automated microtitre broth dilution Merlin MICRONAUT system appears to be superior to other systems (N. Wellinghausen and others, unpublished data). Implementation of automated systems instead of disk diffusion may thus improve the quality of DAST of blood cultures and thereby improve patient care and outcome.

Table 5. Correlation of direct disk diffusion and standard antimicrobial susceptibility test results for Gram-negative bacilli analysed by applying DIN breakpoints

Antibiotic	No. (%) of strains with:			
	SIR agreement*	Very major error	Major error	Minor error
Amoxycillin/clavulanate	134 (60.4)	15 (6.8)	1 (0.4)	72 (32.4)
Ceftazidime	212 (95.1)	2 (0.9)	1 (0.4)	8 (3.6)
Cefuroxime	160 (71.1)	15 (6.7)	1 (0.4)	49 (21.7)
Gentamicin	182 (81.3)	2 (0.9)	2 (0.9)	38 (17.0)
Levofloxacin	217 (96.0)	4 (1.8)	1 (0.4)	4 (1.8)
Piperacillin/tazobactam	202 (90.6)	11 (4.9)	1 (0.4)	9 (4.0)
Total (%)	**1107 (82.4)**	**49 (3.6)**	**7 (0.5)**	**180 (13.4)**

*Category agreement with respect to susceptible (S), intermediate (I) and resistant (R) test results.

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