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Comparison of direct antimicrobial susceptibility testing methods for rapid analysis of bronchial secretion samples in ventilator-associated pneumonia

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ABSTRACT

Two hundred and fifty tracheal aspirates were subjected to direct antimicrobial susceptibility testing by disk diffusion, Etest and inoculation on antibiotic-enriched MacConkey agar plates. Results were compared with those obtained using an automated system on microorganisms recovered from standard quantitative culture. A total of 255 microorganisms were isolated from 194 positive samples by the standard quantitative procedure. A total of 85.1%, 82.5% and 72.5% agreement between direct disk diffusion, Etest and antibiotic-enriched MacConkey agar plates, respectively, and the standard procedure was observed in 64 microorganisms obtained from monomicrobial cultures that corresponded to 240 individual microorganism–antimicrobial agent combinations. Three (1.3%) and four (1.7%) very major errors for direct disk diffusion and Etest methods were observed, respectively. The antibiotic-enriched MacConkey agar plate method compared with the standard procedure demonstrated an unacceptable rate of very major (6.7%) and major errors (14.2%). Clinical evaluation of direct susceptibility tests based on the speculative impact on clinical practice by guiding patient's early treatment, if all positive cultures corresponded to infection, was correct in 79.9% for the direct disk diffusion test, 77.8% for the direct Etest method and 68.0% for antibiotic-enriched MacConkey agar plates. Direct diffusion tests (Etest or disk diffusion) applied on respiratory samples are rapid techniques that provide results comparable with standard antimicrobial susceptibility testing in <24 h.

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1. Introduction

Ventilator-associated pneumonia (VAP) is one of the most frequently observed nosocomial infections in the Intensive Care Unit (ICU) and is associated with high morbidity and mortality. Since administration of appropriate antimicrobial agents is correlated with a decrease in mortality, shortening the period in which empirical therapy is given may result in a better outcome for patients [1–3].

In the ICU of University General Hospital 'Attikon' (Athens, Greece), resistant Gram-negative organisms are responsible for the majority of infections, accounting for ca. 90% of VAP and 50% of bloodstream infections. In this context, colonisation data have been successfully used to guide the administration of empirical antibiotic therapy [4].

In a routine laboratory setting, bacterial identification and susceptibility testing usually take 48–72 h after sampling. Conventional phenotypic methods based on culturing on agar (disk diffusion test and Etest) or on microtitration plates (broth dilution tests) are the most commonly used methods for antimicrobial susceptibility testing (AST).

To shorten the turnaround time required for AST of bacteria, direct disk susceptibility testing has been practiced in some laboratories [5,6], although it is not recommended by the American Society for Microbiology since the inocula is not properly standardised [7]. On the other hand, providing clinicians with early microbiological information becomes beneficial for the patient, permitting proper antibiotic use, less antimicrobial misuse and a decrease in antimicrobial-related adverse events [8].

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Agar diffusion using Etest strips with a predefined stable gradient of antibiotic (AB BIODISK, Solna, Sweden) is a well known AST method that is inoculum size-independent and can be used on isolated bacteria or applied directly to clinical specimens [8–10].

Besides routine culturing, in some laboratories specimens are also streaked on antibiotic-enriched MacConkey agar plates containing antibiotics. Growth on these media during the first 24 h of incubation suggests the presence of resistant pathogens and is reported as a preliminary result to physicians the day after sampling [4,11].

This study compared the results of three direct susceptibility tests for five antimicrobial agents with the respective results of the standard microbiological procedure applied on lower respiratory tract secretions and evaluated their clinical application.

2. Material and methods

This study was performed at the Infectious Diseases Research Laboratory of University General Hospital 'Attikon', in Athens metropolitan area. The hospital has 635 beds and a general (surgical and medical) ICU with 21 beds in use. Empirical antimicrobial treatment in the ICU is guided by biweekly active surveillance of patient flora (faecal, urine and respiratory) [4].

During a 6-month period, bronchial aspirates from patients with suspected VAP [12,13] were examined by the following methods.

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2.1. Method A

Bronchial aspirate samples were diluted with an equal volume of Sputasol (dithiothreitol) (Oxoid Ltd., Basingstoke, UK). Microbial densities of various bacteria were determined by the standard quantitative culture method using 10 μL of saline-diluted samples (10^{-2}, 10^{-4} and 10^{-5}) on Columbia agar supplemented with 5% sheep blood, MacConkey agar and chocolate agar plates [14]. Colonies were counted after 18–24 h of incubation.

All cultures with colony counts of $\geq 10^4$ colony-forming units (CFU)/mL were included and were evaluated for their direct susceptibility testing results, whereas counts $< 10^4$ CFU/mL indicated contamination with oropharyngeal microbiota and were discarded as negative [15]. Although 10^4–10^6 CFU/mL in tracheal aspirates is usually not considered significant, they can potentially predict the aetiology of subsequent infection.

Identification and susceptibility testing of the isolated microorganisms were performed using a BD Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD). Breakpoints for ciprofloxacin, meropenem, piperacillin/tazobactam (TZP) and vancomycin were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines [16], whilst colistin results were interpreted in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [17] as relevant breakpoints were not available from CLSI. All isolates were screened for metallo-β-lactamase (MBL) production by the ethylene diamine tetra-acetic acid (EDTA)–imipenem disk synergy test [18], whilst

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Klebsiella pneumoniae isolates were also submitted to the imipenem–boronic acid disk synergy test as a screening for *K. pneumoniae* carbapenemase (KPC) production [19].

2.2. Methods B and C

After dilution with Sputasol at a proportion of 1:1, samples were directly plated on Muller–Hinton agar plates. Five Etest strips (method B) and five antibiotic disks (method C) (meropenem, colistin, ciprofloxacin, TZP and vancomycin) were placed directly onto the plates and were incubated at 35 °C. Reading of inhibition zones was performed at 18–24 h with transmitted light and results were interpreted according to CLSI and EUCAST susceptibility breakpoints (as previously described). The accuracy of the direct disk susceptibility method for colistin was evaluated using interpretive criteria available from the CLSI for *Pseudomonas aeruginosa* (resistant ≤10 mm and susceptible ≥11 mm), whilst other Gram-negatives (no available criteria from CLSI or EUCAST) were considered resistant when zone diameters were ≤11 mm and susceptible when ≥14 mm. Consequently, zone diameters of 12–13 mm, which according to other publications should be confirmed with minimum inhibitory concentration (MIC) measurement by Etest or broth dilution, were not evaluated [20]. Susceptibilities of *Chryseobacterium indologenes*, *Pseudomonas luteola* and *Achromobacter* spp. isolates were evaluated using MIC interpretive criteria for other non-Enterobacteriaceae available from the CLSI and were excluded from the direct disk susceptibility method evaluation as there are no zone diameter criteria. *Stenotrophomonas maltophilia* isolates were completely excluded from evaluation (no criteria for any of the antibiotics tested).

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2.3. Method D

After dilution with Sputasol at a proportion of 1:1, samples of 10 μL were streaked on antibiotic-enriched MacConkey agar plates containing the following antibiotics: ciprofloxacin (2 mg/L); TZP (32/4 mg/L); meropenem (4 mg/L); colistin (4 mg/L); and vancomycin (6 mg/L). Growth on any of the above media during the first 24 h of incubation suggested the presence of pathogens resistant to the respective antimicrobial agent [4].

Escherichia coli ATCC 25922, *P. aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as control strains in all methods.

Agreements and discrepancies in the results of methods B, C or D with the standard quantitative method A were classified as follows: agreement (identical result); very major error (VME) (susceptible in method B, C or D but resistant in method A); major error (ME) (resistant in method B, C or D but susceptible in method A); and minor error (mE) (susceptible or resistant in method A and intermediate in method B, C or D, or vice versa). The CLSI recommends that <10% mE, <3% ME and <1.5% VME should be obtained to approve the performance of susceptibility tests [21].

Although VAP is mainly a monomicrobial disease, polymicrobial results may occur in no more than 30% of patients with VAP [8]. In the case of polymicrobial results, susceptibility to each antibiotic was determined by the inhibition zone of the less susceptible microorganism.

Methods B, C and D were also evaluated for all samples (monomicrobial and polymicrobial) with the use of the highest MIC, the lowest disk inhibition zone or the existence of growth on each antimicrobial agent. These 'clinical' criteria were based on the speculative impact of the results to correctly guide the choice of empirical treatment in case of infection. The method was determined as correct if the results were in agreement with those of method A or at least one ME was identified, because in this case the clinician would be correctly guided in administering an adequate early treatment to a critically ill patient in sepsis. Antibiotic overtreatment in initial coverage of a septic patient was also considered as correct. Any other result was recorded as a false one because the clinician would be guided to administer an inadequate antibiotic regimen.

3. Results

During the study period (6 months), 250 tracheal aspirates corresponding to 76 patients with suspected VAP were processed by standard quantitative culture (method A) in our research microbiology laboratory. A total of 194 positive samples were recorded, in 67.9% of which the microorganism concentration (colony count) was $\geq 10^5$ CFU/mL, in 14.7% it was 10^4 CFU/mL and in 17.4% it was $\leq 10^3$ CFU/mL. Of these, 64 were monomicrobial and 98 were polymicrobial with $\geq 10^4$ CFU/mL. A total of 255 microorganisms were isolated by the standard quantitative method.

Gram-negative organisms comprised 94.9% of the isolates and belonged to the following species: *Acinetobacter* spp. ($n = 110$); *P. aeruginosa* ($n = 59$); *K. pneumoniae* ($n = 25$); *Proteus mirabilis* ($n = 18$); *S. maltophilia* ($n = 11$); *Providencia stuartii* ($n = 6$); *Enterobacter* spp. ($n = 4$); *E. coli* ($n = 4$); *C. indologenes* ($n = 3$); *P.*

luteola ($n = 1$); and *Achromobacter* spp. ($n = 1$). Gram-positives comprised only the 5.1% of the isolates (10 *S. aureus* and 3 *Enterococcus* spp.).

According to the standard susceptibility testing procedure (method A), colistin was the most active agent against Gram-negative isolates (77.5% susceptible), with ciprofloxacin exhibiting the highest resistance rate (73.7%). Meropenem and TZP exhibited susceptibility rates of 40.7% and 33.6%, respectively. Vancomycin was active against all *S. aureus* isolates, whilst 50.0% were meticillin-resistant. Only one *E. faecalis* was vancomycin-resistant (Table 1).

Among the 59 *P. aeruginosa* isolates, 27 (45.8%) were designated carbapenemase-producers, exhibiting a positive EDTA–imipenem disk synergy test suggestive of MBL production. Among the 25 *K. pneumoniae* isolates, 13 (52.0%) were designated carbapenemase-producers, showing a positive boronic acid–imipenem disk synergy test suggestive of KPC production. Approximately 78% of the *Acinetobacter baumannii* isolates were carbapenem-resistant (Table 1).

Antimicrobial susceptibilities determined by the direct Etest (method B), disk diffusion (method C) and antibiotic-enriched MacConkey agar plates (method D) were compared with the microdilution method (method A) in 64 microorganisms obtained from monomicrobial cultures. Namely, 240 individual microorganism–antimicrobial agent combinations (one microorganism–one antibiotic) were compared in order to detect very major, major and minor errors. A total of 82.5%, 85.1% and 72.5% agreement, respectively, between methods B, C and D and method A was observed. Three (1.3%) very major errors common for both the direct disk diffusion and direct

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Etest methods, corresponding to one *P. aeruginosa* isolate and ciprofloxacin, one *P. aeruginosa* isolate and TZP and one *A. baumannii* isolate and colistin were observed. In addition, one more (total 1.7%) very major error corresponding to an *A. baumannii* isolate and meropenem ($n = 1$) was observed in the Etest method. Fifteen of the sixteen major errors observed in the direct disk diffusion method, corresponding to *P. aeruginosa* and ciprofloxacin ($n = 1$), TZP ($n = 3$) and meropenem ($n = 1$), *E. coli* and ciprofloxacin ($n = 1$), *P. mirabilis* and TZP ($n = 3$) and meropenem ($n = 3$), *K. pneumoniae* and TZP ($n = 1$) and meropenem ($n = 1$) and *A. baumannii* and meropenem ($n = 1$), were also observed in the Etest method. The sixteenth major error corresponded to an *A. baumannii* strain and colistin ($n = 1$). Five more major errors observed in the Etest method corresponded to *A. baumannii* and meropenem ($n = 1$) and colistin ($n = 2$) and *P. aeruginosa* and colistin ($n = 2$).

Method D compared with method A demonstrated an unacceptable percentage of very major (6.7%) and major errors (14.2%) (Fig. 1). Very major errors were detected in *A. baumannii* isolates and ciprofloxacin ($n = 3$), TZP ($n = 2$), meropenem ($n = 5$) and colistin ($n = 2$) and to a lesser extend in *E. coli* and TZP ($n = 1$), *P. aeruginosa* and meropenem ($n = 1$), *K. pneumoniae* and colistin ($n = 1$) and *P. mirabilis* and colistin ($n = 1$).

Clinical evaluation of methods B, C and D based on the speculative impact in clinical practice by guiding patient's empirical treatment was correct in 77.8%, 79.9% and 68.0%, respectively, for 194 positive specimens (Fig. 1). The majority of very major errors (13, 12 and 39, respectively) concerned colistin (in the two diffusion methods) and ciprofloxacin and meropenem (in antibiotic-supplemented agar plates) (Table 2).

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Reliable antimicrobial susceptibility results from methods B, C and D were reported to clinicians in <24 h after pneumonia was clinically suspected, whilst confirmation of susceptibility results were available in 48 h in the case of monomicrobial infection or in 72 h in the case of polymicrobial infection.

Susceptibility testing of quality control strains was performed once weekly with all methods (A, B, C and D). Zone diameter (method C) and MIC values (methods A and B) fell always within the specified quality control limits [16]. None of the quality control strains grew on antibiotic-enriched MacConkey agar plates containing antibiotics (method D).

4. Discussion

Appropriate empirical antimicrobial therapy in sepsis directly affects mortality in the ICU and consists of timely and adequate coverage of the implicated pathogen [22–25]. In settings with high rates of antimicrobial resistance, physicians are obliged to treat critically ill patients empirically using extended-spectrum antibiotics in order to maximise adequacy of coverage, thus contributing to the increase in antimicrobial resistance. De-escalation of initial empirical therapy to a narrow-spectrum antimicrobial regimen and/or a decrease in the number of administered antibiotics is therefore implemented only on the fourth to seventh day after the onset of the infection according to susceptibility test results.

The aim of this study was to find a rapid and easily applicable microbiological method to detect susceptibilities in order to replace initial empirical therapy with targeted

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therapy at an earlier stage. Use of direct susceptibility tests can probably lead to quite accurate results in 24 h compared with standard microbiological methods that are completed at ≥48 h.

The results of a study by Cercenado et al. [10] showed that in patients with VAP, reliable antimicrobial susceptibility data of microorganisms isolated from lower respiratory tract samples can be obtained by direct Etest in <24 h. Their results were comparable with those obtained with the standard procedure, with an overall correlation of 96.1%. On the other hand, it should be considered that the direct disk diffusion technique is cheaper and can deliver reliable early susceptibility results [26]. Many laboratories directly streak an aliquot of all positive blood cultures on agar plates, add antibiotic disks and interpret the inhibition zones after 6–8 h of incubation [7]. However, testing blood culture isolates is easier than testing respiratory samples because the former generally involves a single pathogen, whereas respiratory samples regularly yield more than one pathogen. Application of a direct susceptibility testing technique could help clinicians to administer specific antimicrobial therapy in <24 h after the onset of VAP and, although imperfect, can be beneficial.

In this study, the majority of positive specimens were polymicrobial (66.7%) and most of the isolates were Gram-negatives (94.9%). The percentages of very major errors (1.3%) and major errors (6.8%) in direct disk diffusion technique (method C) were less than in the direct Etest method (method B) (1.7% and 8.75%, respectively) in monomicrobial cultures (Fig. 1). Total agreement was 82.5%, 85.1% and 72.5% for methods B, C and D, respectively. The high rate of errors in method D (6.7% VME,

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14.2% ME and 6.7% mE) considerably restricts its use for direct susceptibility testing of bronchial secretions in patients with VAP.

It is noteworthy that the majority of isolated bacteria in this study were multidrug-resistant isolates (Table 1), with most of the Gram-negatives being susceptible only to colistin. This reality increases the interest in rapid susceptibility testing results, particularly to the latter antimicrobial agent. Agreement in particular of 89.3% and 96.2% for methods B and C for colistin compared with method A suggests that clinicians can rely on this direct susceptibility testing result.

Interpretation of readings was easy in all monomicrobial samples but it was more difficult in polymicrobial ones, in which overgrowth of certain microorganisms prevented the visualisation of others. However, the use of transmitted light as also reported by Cercenado et al. [10] considerably improved the readings.

Interpretation of disk inhibition zones in method C was easier than the inhibition ellipse of the Etest (method B) when the MIC was near the breakpoint. Simultaneous growth of fungi, Gram-positive cocci, mucous Gram-negative strains or resistant subpopulations is the most important factor that prevents the correct determination of MICs by direct susceptibility testing with the Etest (method B).

Nevertheless, in most cases it was possible to visualise different populations with different levels of resistance, as well as in the direct Etest technique the antibiotic concentration that inhibited all microorganisms present in the respiratory sample. Moreover, both direct disk diffusion and direct Etest allow the detection of resistant

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bacteria inside the diameter or ellipse of inhibition. Several studies have evaluated the utility of these two direct methods on blood cultures for determination of the susceptibility or MICs of different antimicrobials. Edelmann et al. [27] reported that the direct disk diffusion method applied to blood cultures exhibited 93.9% agreement with microtitre broth dilution susceptibility testing, with 1.6% very major, 1.1% major and 2.6% minor errors in Gram-positive cocci, whilst in Gram-negative rods agreement was found in 91.9% of cases, with 1.2% very major, 0.7% major and 6.3% minor errors. In another study by Hong et al. [28], Etest proved to be a useful tool to obtain MIC data on Gram-positive cocci (especially streptococci) directly from positive blood cultures. For all isolates tested, a 98.5% concordance rate was achieved between the direct and standard Etests.

All three techniques in the microbiological evaluation of monomicrobial cultures exhibited marginally unacceptable levels of very major and major errors.

If all 194 cultures corresponded to infection, the three techniques would have correctly guided early treatment in 79.9% for method B, 77.8% for method C and 68.0% for method D. Methods B and C can offer a preliminary result for susceptibility evaluation in only 24 h after the suspicion of VAP, but the results must be confirmed by standard susceptibility testing (method A). However, the confirmation can only be achieved after 48 h.

Implementation of direct susceptibility testing in bronchial aspirate samples from patients admitted to the ICU and with suspected VAP is crucial for early modification of therapeutic regimens, which may improve the quality of treatment and thereby

improve patient care and outcome, whilst avoiding resistance development to valuable antibiotics.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Schematic presentation of the study and results. VME, very major error; ME, major error.

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Table 1

Bacterial isolates and their phenotypes recovered from 194 bronchial aspirate

samples tested

Species	Total no. of isolates	Percentage of total isolates (%)
Acinetobacter baumannii	110	43.1
Carbapenem-resistant	86	33.7
Colistin-resistant	11	4.3
Klebsiella pneumoniae	25	9.8
Carbapenemase-producing	13	5.1
ESBL-producing	13	5.1
Colistin-resistant	14	5.5
Pseudomonas aeruginosa	59	23.1
Carbapenemase-producing	27	10.6
Colistin-resistant	0	0
Stenotrophomonas maltophilia	11	4.3
Proteus mirabilis	18	7.1
Other Gram-negatives	19	7.5
Staphylococcus aureus	10	3.9
MRSA	5	2.0
Enterococcus spp.	3	1.2
Vancomycin-resistant	1	0.4

ESBL, extended-spectrum β-lactamase; MRSA, meticillin-resistant *Staphylococcus*

aureus.

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Table 2

Correlation of very major errors (VMEs) and individual antibiotics in clinical evaluation

of direct susceptibility testing

Antibiotic	VMEs		
	Method C (direct disk diffusion)	Method B (direct Etest)	Method D (antibiotic-supplemented agar plates)
Ciprofloxacin	3	5	19
TZP	3	2	11
Meropenem	0	3	19
Colistin	7	6	4
≥1 VME	12	13	39

TZP, piperacillin/tazobactam.

Patients (n=76)

Speciments (n=250)

Negative (n=56)

Monomicrobial (n=84)

Polymicrobial (n=110)

≤10³ cfu/ml (n=20)

≥10⁴ cfu/ml (n=64)

≤10³ cfu/ml (n=12)

≥10⁴ cfu/ml (n=98)

Method B
Direct E-test
VME 1.7%
ME 8.75%

Method C
Direct Disk Diffusion
VME 1.3%
ME 6.8%

Method D
Antibiotic-enriched
Mac-Conkey agar plates
VME 6.7%
ME 14.2%

Not evaluated

Not evaluated

A. baumannii (n=35)
E. aerogenes (n=1)
E. coli (n=1)
K. pneumoniae (n=4)
P. mirabilis (n=6)
P. aeruginosa (n=13)
S. aureus (n=2)
S. maltophilia (n=2)

Achromobacter species (n=1)
A. baumannii (n=75)
C. indologenes (n=3)
E. aerogenes (n=3)
E. faecalis (n=3)
E. coli (n=3)
K. pneumoniae (n=21)
P. mirabilis (n=12)
P. stuartii (n=6)
P. aeruginosa (n=46)
P. luteola (n=1)
S. aureus (n=8)
S. maltophilia (n=9)

Clinical evaluation

Method B
Direct E-test
Correct: 77.8%

Method C
Direct Disk Diffusion
Correct: 79.9%

Method D
Antibiotic-enriched
Mac-Conkey agar plates
Correct: 68%