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Direct antimicrobial susceptibility testing method for analysis of sputum collected from patients with cystic fibrosis

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Abstract

Background: Chronic *Pseudomonas aeruginosa* colonisation and subsequent exacerbations in patients with cystic fibrosis (CF) require antimicrobial treatment. But since multiple morphotypes and other Gram-negative bacteria with different antibiotic susceptibilities are often isolated inside the same sputum sample, bacteriological analysis is difficult.
Methods: To simplify this analysis, we explored a direct sputum antimicrobial susceptibility testing (DSST) method by applying E test directly on plates inoculated with the sputum. A total of 316 samples collected from CF patients were analysed and compared with standard procedures (SP) for the identification and antimicrobial susceptibility testing of all Gram-negative bacterial species.
Results: DSST was as efficient as SP to detect *P. aeruginosa* including the mucoid morphotype in monomicrobial specimen, but was less sensible to detect all Gram-negative bacteria present in the same sample. It allowed the direct reading of the MIC inhibiting all Gram-negative bacteria. Agreements between these global MICs with the cumulative antibiotics susceptibility of all Gram-negative bacteria measured by SP were excellent for tobramycin and imipenem (>96%) and satisfactory for ticarcillin, ceftazidime, aztreonam and ciprofloxacin (90.4% to 94.3%).
In conclusion, the DSST method is an efficient and easy antibiotic susceptibility testing method.
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Keywords: E test; Overall MIC; Cystic fibrosis

1. Introduction

Pseudomonas aeruginosa is the most frequent and important endobronchial pathogen associated with mortality and morbidity in patients with cystic fibrosis (CF). Antimicrobial therapy has made an important contribution to increase the life expectancy of CF patients. Because of the frequent administration of antibiotics, multiresistant strains are increasingly discovered within the *P. aeruginosa* species. These bacteria with different susceptibility patterns associated to multiple colonies morphotypes like mucoid or small colony variants of *P. aeruginosa* are often isolated in the same sputum sample. Other Gram-negative bacteria such as *Burkholderia cepacia* complex organisms, *Stenotrophomonas maltophilia*, or *Achromobacter xylosoxidans* have also emerged due to their innate resistance to many antibiotics. We have to screen all of them and study their susceptibility to antibiotics, even if the pathogenic significance (ie *S. maltophilia*) is not fully clarified for some of them [1–3].

The principal difficulty in the analysis of CF patients samples is to identify all different morphotypes of *P. aeruginosa* and other microorganisms and to study their antimicrobial susceptibility in view to guide antibiotherapy. This analysis is currently based on the antibiotic susceptibility determining of each morphotype of *P. aeruginosa* and Gram-negative bacterial isolates. This method is complex, fastidious and time-

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consuming. Furthermore, we can miss to test some antibiotics-resistant *P. aeruginosa* clones within a same sputum sample.

The aim of this multicenter study was to assess a new method for a simple and efficient determination of the antimicrobial susceptibility of all Gram-negative bacteria. This method is performed by the application of gradients of antipseudomonal antibiotics (E test® strips, AB Biodisk, Solna, Sweden) directly on agar plates inoculated with the sputum. This method, referred to as direct sputum antimicrobial susceptibility testing (DSST), provided an overall "MIC" on the Gram-negative flora. This overall MIC was compared to the cumulative results of antimicrobial susceptibility performed against each isolates with standard procedures (SP). Performances of this method with regard to detection of all pathogens were also analysed.

2. Materials and methods

2.1. Patients and clinical specimens

Three hundred and sixteen sputum samples were included in this study. They were recovered from 96 adults (mean age: 23.5 years; range: 18–32) and 41 children (mean age: 14 years; range: 5–18) known to be chronically infected by *P. aeruginosa* alone or in association with other microbial species. Many samples could be collected from the same patient during the study time. They harboured either the same predominance of pathogens with the same antibiotics pattern or harboured different patterns of resistance, bacterial species and different rates of colonisation since patients received antibiotic treatments during this time. In

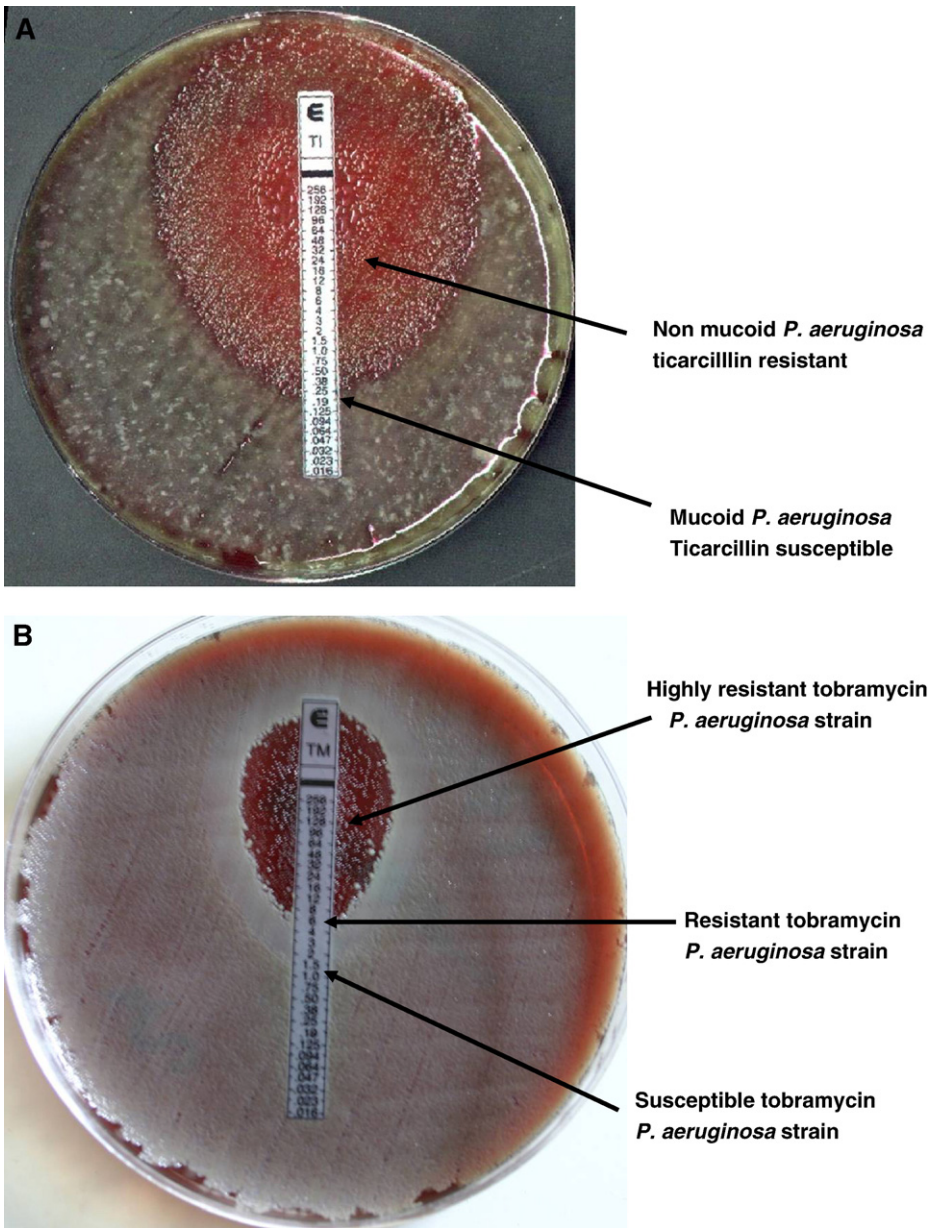

Fig. 1. Cumulative percentage of global MICs (mg/mL) of ticarcillin, ceftazidime, imipenem, aztreonam, tobramycin and ciprofloxacin inhibiting all Gram-negative bacteria.

Table 1
Comparison of antimicrobial susceptibilities measured by DSST and by standard procedures (SP) according to each antibiotic

Antibiotic	Comparison of the susceptibility results obtained by DSST and SP			
	Agreement (%)	Very major discordance (%)	Major discordance (%)	Minor discordance (%)
Ticarcillin	90.4	3.2	4.8	1.8
Aztreonam	94.3	3.7	2	0
Imipenem	96.3	2.2	0.8	0.7
Ceftazidime	92.3	2.5	2.1	3.1
Ciprofloxacin	91.7	6.6	1.1	0.7
Tobramycin	96.1	1.3	0.7	1.9

Results were interpreted as follows: Agreement: DSST = SP. Very major discordance: DSST sensitive and SP resistant; major discordance: DSST resistant and SP sensitive; minor discordance: DSST I and SP resistant or susceptible or SP intermediate and DSST resistant or susceptible.

the first case, we could check the reproducibility of our results and in the second we controlled the evolution of microbial flora. Bacterial analysis was processed using the same procedures in the 4 Cystic Fibrosis Centre-dependant laboratories.

2.2. Direct sputum antimicrobial susceptibility testing (DSST)

Sputum samples were solubilized in *N*-acetylcystein (vol/vol), diluted to 1/20 in saline and swabbed on blood-supplemented Mueller–Hinton agar plates (BioMérieux, Marcy L'Etoile, France). E test strips of the following six antibiotics: ticarcillin, ceftazidime, imipenem, aztreonam, tobramycin and ciprofloxacin, were placed directly onto different agar plates. The minimal concentration of the antibiotic that inhibited the totality of Gram-negative organisms was determined by reading after 18, 24 and 48 h of incubation the antimicrobial concentration printed on the E test strip at its intersection with the growth inhibitory zone of all Gram-negative isolates according to the recommendations of the manufacturer. The reading of the global MICs was checked by 2 different persons. Bacterial identification was performed as expected in all different colonies.

2.3. Standard procedures (SP)

A calibrated aliquot (100 µl) of such solubilized sputum and its dilution (1/100 in saline water) were set up for quantitative culture on a panel of non-selective and selective media (BioMérieux, Marcy L'Etoile, France) including at least Colombia colistin–nalidixic acid agar (CNA), *B. cepacia*-selective medium [3] and cetrimide agar. After 18 to 24 h of incubation, all colonies corresponding to the same bacterial species were counted. Identification of organisms was performed by means of standard microbiological tests including API 20 NE strips (bioMérieux, Marcy l'Etoile, France). If necessary, partial 16SrRNA gene sequencing was used for the identification of atypical non-fermenting Gram-negative bacteria. A threshold of 10^2 CFU/mL for *P. aeruginosa* and *B. cepacia* and of 10^5 CFU/mL for the other species was considered significant.

Antimicrobial susceptibility testing was performed on each morphotype of *P. aeruginosa*, as well as on other Gram-negative non-fermenters using the disk diffusion test on Mueller–Hinton agar (BioMérieux, Marcy L'Etoile, France). The inhibition zone diameters were measured after 18 to 24 or 48 h of incubation to accommodate slower-growing strains, and susceptibility interpreted using NCCLS criteria now CLSI [4].

2.4. Interpretation of results

The results obtained by DSST were compared to the cumulative susceptibility of all morphotypes of *P. aeruginosa* and other

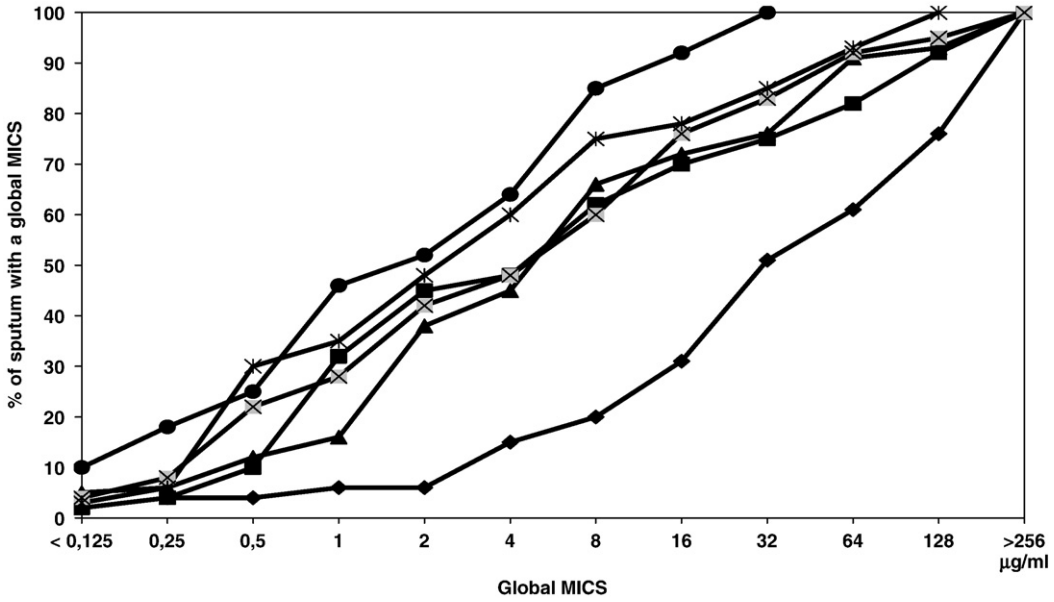

Fig. 2. Cumulative percentage of global MICs (mg/mL) of ticarcillin, ceftazidime, imipenem, aztreonam, tobramycin and ciprofloxacin inhibiting all Gram-negative bacteria.

bacterial species measured by SP and interpreted as susceptible to an antibiotic when all bacterial strains were susceptible and resistant to an antibiotic only when one morphotype of *P. aeruginosa* or another Gram-negative bacterial species was resistant. DSST susceptibility results defined as "observed" susceptibility were compared to the "predicted" susceptibility inferred from the most resistant cumulative pattern of all individual *P. aeruginosa* morphotypes and other Gram-negative bacteria. Discrepancies between the "predicted" and "observed" susceptibilities were classified as very major (VM) if "predicted" resistant, "observed" susceptible, major (M) if "predicted" susceptible, "observed resistant", minor (MN) if "predicted" or "observed" intermediately susceptible, other results susceptible or resistant.

3. Results

3.1. Microbiology of the sputa

The analysis of the Gram-negative flora by SP showed that *P. aeruginosa* was recovered from 303 of the 316 sputa (95.8%). The bacterial load was ≥ to 10^5 CFU/mL for 276 of them (91%). Six hundred and fifty-one mucoid or non-mucoid morphotypes, with different antibiotics susceptibility patterns of *P. aeruginosa*, which result in a mean of 2.2 (range 1–4) morphotypes per specimen, were differentiated. *P. aeruginosa* was the sole pathogen in 241 (79.5%) of sputa and was associated either with *S. aureus* in 32 sputa (10.5%) or other Gram-negative bacteria in 30 sputa (10%). A total of 48 strains of non-*P. aeruginosa* Gram-negative non-fermentative bacteria have been isolated. They belonged to the following species: *A. xylosoxidans* (n=17), *B. cepacia* (n=12), *S. maltophilia* (n=12), *Bordetella bronchiseptica* (n=3), *Sphingomonas paucimobilis* (n=2), *Sphingobacterium multivorum* (n=1) and *Ochrobactrum anthropi* (n=1).

Table 2
Comparison of the ability of standard procedures (SP) and DSST to detect Gram-negative non-fermentative organisms in 316 CF sputa

Composition of the Gram-negative bacteria from SP		Performance of DSST for the detection of	
Bacterial species	*P. aeruginosa* rate of colonisation	*P. aeruginosa*	GNFB
P. aeruginosa alone (n[a]=241)	$<10^5$ CFU/mL (n=21)	11 (52.4%)	
	$>10^5$ CFU/mL (n=220)	220/220 (100%)	
P. aeruginosa+ *S. aureus* (n=32)	$<10^5$ CFU/mL (n=3)	2/3	
	$>10^5$ CFU/mL (n=29)	24/29 (82.7%)	
P. aeruginosa+ other GNFB (n=30)	$<10^5$ CFU/mL (n=2)	0/2	2/2
	$>10^5$ CFU/mL (n=28)	24/28 (85.7%)	16/28 (57%)
Other GNFB (n=13)			
One GNFB alone (n=8)			One GNFB: 7/8 (87.5%)
Two GNFBs associated (n=5)			Two GNFBs: 3/5 (60%)

[a] n=number of sputa.

3.2. Accuracy of antimicrobial susceptibility testing

Fig. 1 showed direct E test on sputum sample containing different morphotypes and susceptibility levels *P. aeruginosa*. Minimal concentrations of antibiotic inhibiting the totality of Gram-negative bacilli was measured by reading after 18, 24 and 48 h of incubation the antimicrobial concentration printed on the E test strip at its intersection with the growth inhibitory zone of all Gram-negative bacteria. They were compared to the cumulative antibiograms performed on each morphotype of *P. aeruginosa* and other Gram-negative organism in Table 1. Categorical agreements varied from 90.4% to 96.3% according to the antibiotic. The best correlations between "predicted" and "observed" susceptibilities were observed for tobramycin and imipenem (>96%). On the contrary, the highest rate of VM discrepancies was observed for ciprofloxacin (6.6%). Finally, the rate of agreement was better for aztreonam (94.3%) and ceftazidime (92.3%) than for ticarcillin (90.4%).

The distribution of the global minimal concentrations of antibiotics inhibiting the all Gram-negative bacteria present in the sputum was summarized in Fig. 2. This distribution demonstrated a great variability in MICs of antibiotics. Within susceptible β lactams or tobramycin strains, MICs could vary from very low values as 0.128 μg/mL to values near the breakpoints (4 or 8 μg/mL). The same range was observed for resistant strains with values from 32 mg/L to over 256 μg/mL.

3.3. Accuracy of detection of microorganisms

Performances of DSST to screen *P. aeruginosa* and the other Gram-negative bacteria were compared to SP in Table 2. DSST was as sensitive as SP for the detection of *P. aeruginosa* when it was the sole pathogen and present at a rate of colonisation >105 CFU/mL. This method was also as effective as SP to recognize the different morphotypes of *P. aeruginosa* (data not shown). However, the rate of *P. aeruginosa* detection by DSST decreased to 85.7%, when *P. aeruginosa* was associated with another Gram-negative organism, to 82.7% when it was associated with *S. aureus* and further more (52.4%) if the rate of colonisation was low. DSST was as efficient as SP to detect the non-*P. aeruginosa* Gram-negative from monomicrobial specimen (87.5%), but it failed in this way when many bacterial species were mixed in the same sample (60%). Nevertheless, one of the *P. aeruginosa* and *A. xylosoxidans* colonisations was only detected by means of DSST thanks to gradients of antibiotics.

Finally, the comparison of the performances of the DSST method did not show any significant inter-laboratory variations among the 4 participating laboratories (data not shown).

4. Discussion

Selection of the most appropriate antibiotic therapy is essential for the treatment of bronchial exacerbations in CF patients. Standard methods of isolating and testing individual colony types from a sputum are expensive, time-consuming, and they don't ensure the detection of the most resistant organisms. Two strategies have been

proposed to simplify this analysis. The first one is based on the use of antibiotic-containing media, allowing the detection of organisms exhibiting innate or acquired resistance to these antibiotics. Van Dalfsen et al. [5] demonstrated that a tobramycin-containing medium was more sensitive than the standard detection method for the detection of tobramycin-resistant *P. aeruginosa*, *S. maltophilia* and *A. xylosoxidans* strains. Besides, preparation of selective media with antibiotics such as imipenem or colistin has been proposed to detect these specific Gram-negative bacterial species from their antibiotic innate resistance [6]. The second one is to analyse susceptibility tests on *P. aeruginosa* using a mixed-morphotype inoculum. Correlations of these methods performed on mixed strains with broth microdilution susceptibility or disk diffusion methods performed on individual morphotypes were of 96 and 92.2% respectively [7,8].

In the present study, we evaluated a new approach by determining the global MIC of 6 antipseudomonal compounds on all Gram-negative bacteria by placing E test strips directly on agar plates inoculated with sputum. This method is based on the properties of E test which is an inoculum-tolerant and drug stable system and allows thus right MICs determination [9]. This method was assessed by comparing global MIC results with the cumulative susceptibility of all bacterial strains measured using the disk diffusion test. Agreement was excellent for tobramycin and imipenem (>96%) and satisfactory (90.4% to 94.3%) for the other antibiotics tested. Resistance to ciprofloxacin appeared however underestimated by DSST in 6.6% of cases. This discrepancy was due to a majority of borderline strains for which MICs were near the breakpoints defining resistance or susceptibility. The superiority of DSST over standard procedures with regard to the detection of resistant strains defining here as M discordance was demonstrated in 0.7 to 4.8% of cases. We don't know if the resistant *P. aeruginosa* clones are potentially more pathogens than susceptible clones. But this method is more representative of what is going on in the lung than SP and has certainly better relevance in clinical outcome. The other potential advantages of this method were to obtain results within 48 h rather 3 to 4 days with SP and to provide a simple microbiological interpretation by reporting only the overall concentrations of antibiotic inhibiting all Gram-negative bacteria. This method could help the clinicians for screening the most efficient antibiotic by comparing MICs or checking antibiotic resistance by following MICs increase.

DSST method performances were also evaluated in regard to its power to detect *P. aeruginosa* including mucoid morphotypes and other non-fermentative bacteria colonisation. DSST was proven to be reliable essentially when *P. aeruginosa* was the sole pathogen isolated and when colonisation was heavy ($>10^5$ CFU/mL). Co-colonisation with *S. aureus* or other multiresistant organisms, such as *S. maltophilia* or *A. xylosoxidans*, which are not clearly associated with clinical deterioration, might lead to an underestimation of *P. aeruginosa* colonisation by covering it when such a global test on the whole Gram-negative flora was performed. This new method failed also in the detection of all Gram-negative species when they were mixed in the same sample. Thus the DSST method could not serve as substitute for SP to check low colonisations of the main species *B. cepacia* and *P. aeruginosa* and to define the right composition of the flora. For these requests, we recommend to check the low colonisations of the two main species by isolating undiluted sputum on selective media for each sample analysed by DSST and the presence of the other Gram-negative bacteria by performing alternatively bacteriological analysis by SP even if they are not clearly associated with clinical deterioration.

Our method is currently efficient only for Gram-negative bacteria and cannot be applied to *S. aureus* for which the antibiotic susceptibility is different. But, colonies of *S. aureus* could be easily detected on agar plates used for DSST, and we could determine its antimicrobial susceptibility by a standard susceptibility test.

At the present time, the treatment of *P. aeruginosa* infection consists of a combination of 2 or 3 antibiotics. The checkerboard technique is the usual method to determine synergism. Recently, many works have reported the use of E test to assess the activity of antimicrobial combinations by superposing E test strips and comparing MICs of antibiotics tested alone in association. Agreement between the checkerboard method and the E test was 90% for *B. cepacia* [10] and superior to 90% for *P. aeruginosa* strains included mucoid isolates from cystic fibrosis patients [11]. The aim of this current study was to evaluate the accuracy of a direct susceptibility test on CF sputum using E test, but our results support the further evaluation of the E test to apply this method directly on CF sputum.

In conclusion, DSST is an efficient alternative method for rapidly determining the susceptibility of all Gram-negative bacterial species recovered from sputum of CF patients and to follow their resistance by controlling any increase in the MICs of antibiotics. It represents a lower cost by limiting manipulations and the number of antibiotics tested. It improves the interpretation of the bacteriological sputum analysis and allows the detection of all resistant bacteria. This request is appropriate for the main antibiotics and especially for tobramycin in patients receiving inhaled tobramycin. Further clinical trials should now be performed to establish whether the use of the DSST method and its application to antibiotics associations could lead to better therapeutic results.

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