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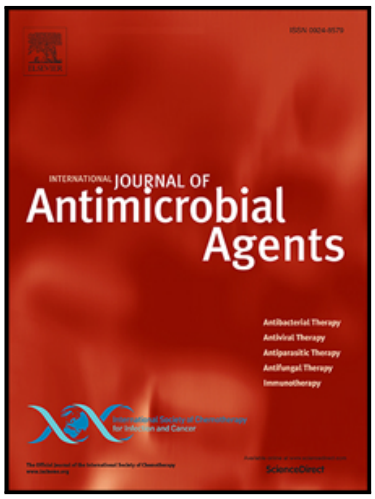

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Highlights

- Non-fermenters other than *P. aeruginosa* are increasingly isolated from cystic fibrosis patients
- Cotrimoxazole is the most active compound against these pathogens
- All the microorganisms presented either a colistin MIC_{90} higher than 16 mg/L
- No statistically significant differences in susceptibility rates between countries

Antimicrobial susceptibility of non-fermenting Gram-negative pathogens isolated from cystic fibrosis patients

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Abstract

Non-fermenting Gram negative bacteria are increasingly being cultured in respiratory samples from people with cystic fibrosis (CF)**.** This study aimed to determine the susceptibility of clinical CF respiratory isolates from distinct geographical regions to a range of antimicrobials**.** A total of 286 isolates (106 *Stenotrophomonas maltophilia*, 100 *Burkholderia* spp., 59 *Achromobacter* spp., 12 *Pandoraea* spp. and 9 *Ralstonia* spp.) from The Netherlands, Northern Ireland, Spain, USA and Australia were tested. The MIC_{50}, MIC_{90} and susceptibility categorization were determined. Cotrimoxazole was the most active compound for all the microorganisms (MIC_{50}=0.12-4 mg/L, MIC_{90}=1-16 mg/L). For *S. maltophilia*, 47% and 62% of

isolates were susceptible to cotrimoxazole according to CLSI and EUCAST breakpoints, respectively. Ceftazidime presented a lower level of susceptibility (35%, MIC_{50}=32 mg/L, MIC_{90}=256 mg/L). Tobramycin and colistin MIC_{90} were >128 mg/L and >16 mg/L, respectively. Regarding *Burkholderia* isolates, 72%, 56% and 44% were susceptible to cotrimoxazole, ceftazidime and meropenem, respectively. For both ceftazidime and meropenem, the MIC_{50} and MIC_{90} values were within the intermediate or resistant category. The most active antibiotics for *A. xylosoxidans* were cotrimoxazole (MIC_{50}=2, MIC_{90}=8 mg/L) and imipenem (MIC_{50}=2, MIC_{90}=8 mg/L). Cotrimoxazole, imipenem, and ciprofloxacin were active against the 12 *Pandoraea* species (MIC_{50}= 0.12-4 mg/L, MIC_{90}= 1-8 mg/L). Ciprofloxacin (MIC_{50}=4 mg/L) and cotrimoxazole (MIC_{50}=1 mg/L) were the only active antibiotics for *Ralstonia* spp. There were no statistically significant differences in susceptibility rates between countries.

Non-fermenting Gram-negative bacteria other than *P. aeruginosa* are potential pathogens in CF and cotrimoxazole has demonstrated to be the most active compound against them.

Keywords: cystic fibrosis, Gram-negative non-fermenting bacteria, antimicrobial susceptibility

Introduction

Cystic fibrosis (CF) patients are regularly colonized by opportunistic microorganisms. In adult patients, *Pseudomonas aeruginosa* is the main pathogen, but in recent years, other non-fermenting bacteria from different genera, such as *Stenotrophomonas*, *Burkholderia*, *Achromobacter*, *Ralstonia*, and *Pandoraea*, are increasingly being isolated [1–3]. This could be potentially due to the aggressive antimicrobial therapy used against *P. aeruginosa*, to the apparition of new techniques for the bacterial identification and improvement in life expectancy for people with CF [4].

In most cases, it has been reported that chronic colonization with these microorganisms is associated with a decline in pulmonary function; however, the pathogenic role of some of these species is not completely clear due to their coexistence with other pathogens including

Staphylococcus aureus and *P. aeruginosa* [4]. Unlike *P. aeruginosa*, for which antimicrobial treatment is standardized, treatment protocols are not in place for these non-fermenting microorganisms and limited data is available regarding their susceptibility profiles. Studying the susceptibility patterns and the epidemiology of these microorganisms is essential to improve the management of CF patients.

The aim of this study was to determine the susceptibility of CF non-fermenting Gram-negative bacteria other than *P. aeruginosa* to a range of antimicrobials used in CF patients. This study is included within the objectives of the IMI-iABC European project which is mainly focused on the development of a new inhaled compound for CF patients.

Material and Methods

A total of 286 isolates recovered from respiratory samples of CF patients (2003-2016) from 5 different countries [Spain (n=103), Northern Ireland (n=98), The Netherlands (n=82), USA (n=2) and Australia (n=1)] were included in the study. Overall, 106 *Stenotrophomonas maltophilia*, 100 *Burkholderia* spp. (51 *B. multivorans*, 20 *B. cenocepacia*, 12 *B. contaminans*, 10 *B. vietnamensis*, 4 *B. cepacia*, 3 *B. gladioli*), 59 *Achromobacter* spp. (53 *A. xylosoxidans*, 4 *Achromobacter* sp., 2 *A. insolitus*), 12 *Pandoraea* spp. and 9 *Ralstonia* spp. isolates were studied. These bacteria were identified by MALDI-TOF MS; the identification was confirmed by whole genome sequencing. The species of *Burkholderia* were also confirmed by sequencing the *recA* gene as previously described [5].

MIC's were determined by standard ISO broth microdilution method with frozen panels (Trek Diagnostic Systems, Westlake, Ohio). The antibiotics and its concentration range tested were: ciprofloxacin (CIP, 0.03-32 mg/L), tobramycin (TOB, 0.125-128 mg/L), ceftazidime (CAZ, 0.25-256 mg/L), meropenem (MER, 0.06-64 mg/L), imipenem (IMI, 0.125-128 mg/L), aztreonam (AZT, 0.25-256 mg/L), cotrimoxazole (SXT, 0.06-32 mg/L), and colistin (COL, 0.25-16 mg/L).

MIC_{50}, $MIC_{90,}$ and susceptibility categorization were assessed considering both EUCAST and CLSI breakpoints and the epidemiological cutoff (ECOFF) when available (Table 1, Table 2, Figure 1). To analyze the data by country**,** chi-square/Fisher test with the Bonferroni correction was used in order to compare susceptibility rates when an antibiotic breakpoint was available (statistically significant when $P<0.016$). To reflect differences in mechanisms of antibiotic resistance between countries, MIC_{90} values were graphically represented (Figure S1).

Statistical analysis was performed using STATA statistical software for Windows (Data Analysis and Statistical Software, version 11.0). Isolates categorized as intermediate and susceptible were grouped for data analysis.

Results

MIC_{50} and MIC_{90} results for all the tested antibiotics and microorganisms are presented in Table 1. SXT was the most active compound for all the microorganisms, with a MIC_{50} range of 0.12-4 mg/L, and a MIC_{90} range of 1-16 mg/L. All the microorganisms presented either a COL MIC_{50} or MIC_{90} higher than 16 mg/L.

Analysing *S. maltophilia* isolates, 47% and 62% of the isolates were susceptible to SXT when considering CLSI ($S\leq2$, $R\geq4$ mg/L) and EUCAST ($S\leq4$, $R>4$ mg/L) clinical breakpoints, respectively (Table 2, Figure 1). Coinciding with CLSI breakpoint, the EUCAST SXT ECOFF is 2 mg/L, which means that 47% of the isolates were included in the wild-type population. CAZ presented a lower level of susceptibility (S 35%; I 6%; R 59%), placing both, the MIC_{50} (32 mg/L) and MIC_{90} (256 mg/L) within the resistant population (Table 2, Figure 1). There are not defined clinical breakpoints for CIP, but the MIC_{50} value (4 mg/L) was close to the EUCAST modal value (2 mg/L) in *S. maltophilia*. For both tobramycin and colistin, MIC_{90} values were high (>128 mg/L, >16 mg/L, respectively).

Considering all the *Burkholderia* species, according to CLSI breakpoints, 72%, 56% and 44% of the isolates were susceptible to SXT (S≤2, R≥4 mg/L), CAZ (S≤8 mg/L, I=16 mg/L, R≥32 mg/L) and MER (S≤4 mg/L, I=8 mg/L, R≥16mg/L), respectively (Table 2). For both CAZ and MER, the MIC_{50} and the MIC_{90} were included within the intermediate or resistant category. Although the MIC_{90} was lower for MER (32 mg/L) than for CAZ (128 mg/L).

Analysing the antimicrobial activity of the different species of the *Burkholderia cepacia* complex (51 *B. multivorans*, 20 *B. cenocepacia*, 12 *B. contaminans*, 10 *B. vietnamensis* and 4 *B. cepacia*), the lowest CAZ MIC_{90} was obtained for *B. vietnamensis* (16 mg/L vs. 64-256 mg/L). *B. vietnamensis* also presented lower IMI MIC_{50} (1 mg/L) and MIC_{90} (32 mg/L) values, than the other species [(IMI MIC_{50} (32-64 mg/L), MIC_{90}128-256 mg/L) range]. The susceptibility profiles for the rest of the antibiotics were similar for all the *Burkholderia* species.

Neither CLSI nor EUCAST have established specific clinical breakpoints for *Achromobacter* spp. Different *Achromobacter* species were not analysed separately as the majority of isolates tested were *A. xylosoxidans* (91.4%). After SXT, the most active antibiotic was imipenem (IMI MIC_{50}=2, MIC_{90}=8 mg/L). The COL MIC had a bimodal distribution, with a first modal value of 1 mg/L and a second modal value of 32 mg/L.

SXT, IMI, and CIP were active against the 12 *Pandoraea* isolates tested, with a range of MIC_{50} and MIC_{90} values of 0.12-4 mg/L and 1-8 mg/L, respectively. Analysing *Ralstonia* spp., a high level of antimicrobial resistance was observed, with CIP (MIC_{50}=4 mg/L) and SXT (MIC_{50}=1 mg/L) as the only active antibiotics.

For *S. maltophilia*, there were no statistically significant differences between countries in CAZ and SXT susceptibility ($p>0.016$) as shown in Figure S1, where the antibiotic MIC_{90} values are reported for the separate countries.

Considering the origin of *Burkholderia* species, *B. multivorans* was most frequently isolated species in Northern Ireland and The Netherlands (48.6% and 68.6%, respectively) while in Spain it was *B. contaminans* (39.3%). A lower rate of MER susceptibility was observed for

Burkholderia isolates from Northern Ireland (54.3%) compared with those from Spain (78.6%) and The Netherlands (74.3%) although it was not statistically significant (p=0.02). Curiously similar MER MIC_{90} values were observed between these countries (4-8 mg/L) (Figure S1). There were no statistically significant differences in CAZ and SXT susceptibility by country, although CAZ MIC_{90} for isolates from Spain was 2-3 dilutions lower than the CAZ MIC_{90} found in The Netherlands and Northern Ireland. The SXT MIC_{90} for isolates from Northern Ireland was 2-3 dilutions lower than that found from Spain and The Netherlands.

For *Achromobacter* spp., SXT MIC_{90} value reported from Northern Ireland was 4 dilutions lower than that from Spain.

Discussion

Non-fermentative Gram-negative bacteria other than *P. aeruginosa*, such as *Burkholderia cepacia complex*, *Stenotrophomonas*, *Achromobacte*r, *Ralstonia*, and *Pandoraea*, are increasingly isolated in respiratory samples from CF patients. These microorganisms are in general intrinsically resistant to multiple antibiotics and treatment guidelines are not yet available, so clinicians judge each patient individually considering the *in vitro* antimicrobial susceptibility reports and clinical outcome after therapy. Furthermore, the available clinical breakpoints are intended for systemic therapy and may not be adequate for inhaled therapy. Inhaled therapy has the potential to achieve high pulmonary concentrations and may be able to inhibit microorganisms with MICs above the breakpoints for systemic therapy [6].

Some studies have defined *S. maltophilia* as a colonizer, while others demonstrated that this microorganism is capable of causing a deterioration in pulmonary function [7,8]. Nevertheless, the presence of *S. maltophilia* cannot be ignored in some patients, as it is associated with an increased risk of pulmonary exacerbations, the need for lung transplantation, and death [9]. Generally, SXT is the antibiotic of choice, but during the last years increasing resistance rates are being reported in CF patients ranging from 16% to 45% [2,3,10,11]. In our

study, the SXT resistance rate was 38% and 53% following EUCAST and CLSI guidelines, respectively. Comparison between EUCAST MIC distribution and that obtained in this study shows a clear displacement of the latter to higher concentrations. In fact, the majority of non-CF *S. maltophilia* isolates are susceptible to SXT (global rate of less than 10% resistance) [12]. Although CAZ and fluoroquinolones are considered as options for *S. maltophilia*, high resistance rates of both compounds are also increasingly being reported. In our study, approximately 60% of the isolates were CAZ resistant, a rate similar to that previously published (80% [13], 70% [2]). Newer fluoroquinolones, such as moxifloxacin may have a better activity against *S. maltophilia* than CIP (MIC_{90} = 32 mg/L in this study) [12].

The isolation of *Burkholderia* spp. is particularly worrying in CF patients, as it is related to a rapid decline in pulmonary function and to a high morbidity and mortality [14]. Similar to *S. maltophilia*, SXT is the antibiotic of choice for *Burkholderia* spp., but combinations are frequently used. A lower SXT resistance rate (28%) was observed than for *S. maltophilia*. Resistance rates for MER and CAZ were more than 30% for both antibiotics.

The clinical relevance of the isolation of species of *Achromobacter* in the sputum of CF patients is unclear. Some studies have demonstrated that its presence is associated with a risk of pulmonary exacerbation but not with a worsened long-term prognosis [15]. Also, *Achromobacter* isolated from people with CF seem to be more virulent than those isolated from other sources [16]. The most active agents for *Achromobacter* were SXT and IMI. In contrast to previously published data, MER was less active than IMI [16]. CAZ, COL, and TOB have been considered adequate for inhalation therapy [17]; however, in this study, these antibiotics presented high MIC_{90} values.

The prevalence of *Ralstonia* and *Pandoraea* infection in CF is low. The pathogenic role of *Pandoraea* spp. appears to be due to the increase in the production of pro-inflammatory cytokines, but the clinical impact is still uncertain[18]. Although only a limited number of *Ralstonia* and *Pandoraea* isolates were tested, our results demonstrated that IMI and SXT had

good activity against *Pandoraea* and CIP and SXT against *Ralstonia*. These observations agree with previously published data [4,18,19].

As the scope of the initial research of the iABC project was to analyze *P. aeruginosa* susceptibility, a limitation of our study is that some antibiotics suitable for non-fermenters other than *P. aeruginosa* were not included in the MIC panels. However, the findings of our study provide insights about the epidemiology and susceptible patterns of these microorganisms from different geographical regions.

In conclusion, non-fermenting Gram-negative bacteria other than *P. aeruginosa* are potential pathogens increasingly being isolated from respiratory samples of CF patients. Little is known about their epidemiology, clinical management and antimicrobial susceptibility. We provide susceptibility testing data to different antimicrobials to better define their antimicrobial susceptibility profile. Considering available clinical breakpoints, SXT has demonstrated to be the most active compound against all isolates tested.

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Declarations

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Table 1. MIC_{50} and MIC_{90} results for the tested antibiotics and microorganisms

	CAZ mg/L	MER	IMI	AZT	CIP	TOB	COL	SXT
S. maltophilia (n=106)								
MIC_{50}	32	>64	>128	>256	4	64	1	4
MIC_{90}	256	>64	>128	>256	32	>128	>16	16
***Burkholderia* spp.** (n=100)								
MIC_{50}	8	8	32	128	4	128	>16	2
MIC_{90}	128	32	128	>256	64	>128	>16	8
***Achromobacter* spp.** (n=59)								
MIC_{50}	8	2	2	>256	8	128	2	0,5
MIC_{90}	128	32	8	>256	32	>128	>16	8
***Pandoraea* spp.** (n=12)								
MIC_{50}	128	>64	2	>256	4	128	>16	0,12
MIC_{90}	256	>64	8	>256	8	>128	>16	1
***Ralstonia* spp.** (n=9)								
MIC_{50}	16	128	32	> 256	4	128	> 16	1

CAZ (ceftazidime), MER (meropenem), IMI (imipenem), AZT (aztreonam), CIP (ciprofloxacin), TOB (tobramycin), COL (colistin), SXT (cotrimoxazole).

Table 2. Clinical susceptibility of *S. maltophilia* and *Burkholderia* spp. to ceftazidime, meropenem and cotrimoxazole

		CAZ			MER			SXT	
	(%)	S	I	R	S	I	R	S	R
CLSI	*S. maltophilia*	35	6	59	-	-	-	47	53
	Burkholderia spp.	56	5	39	44	25	31	72	28
EUCAST	*S. maltophilia*	-[a]	-	-	-	-		62	38
	Burkholderia spp.	-	-	-	2	98	-	-	-

[a]Clinical breakpoints have not been defined

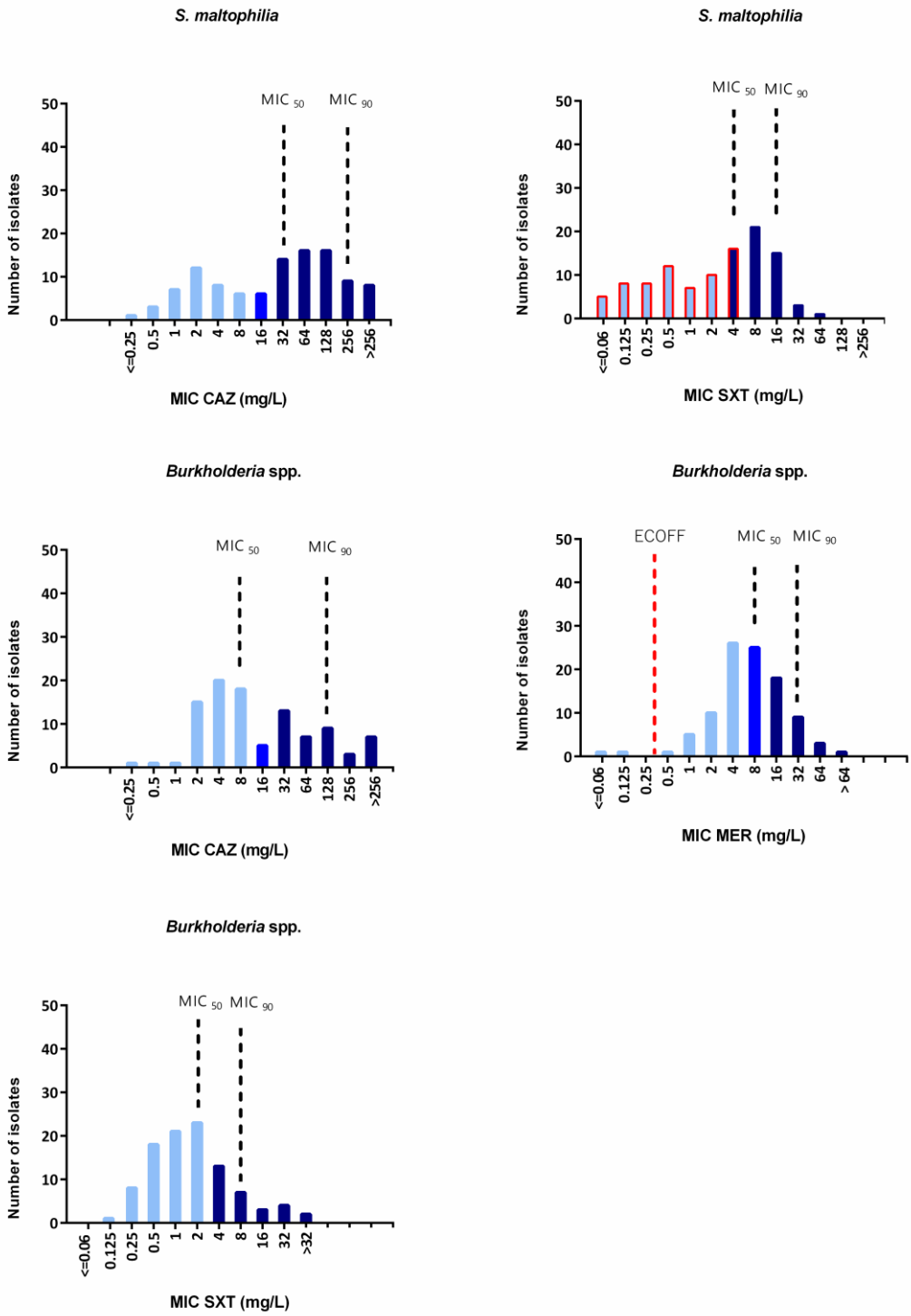

Figure 1. Ceftazidime, cotrimoxazole and meropenem MIC distribution for *S. maltophilia* and *Burkholderia* spp. Susceptibility categorization was performed using the CLSI guidelines (Susceptible: light blue, Intermediate: medium-light blue and resistant: dark blue). For *S. maltophilia* and cotrimoxazole, EUCAST susceptible isolates are marked in red. In the case of *Burkholderia* and meropenem, the ECOFF is represented by a discontinuous red line.