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EuroCareCF Quality Assessment of Diagnostic Microbiology of Cystic Fibrosis Isolates▽

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The identification of microbial species from respiratory specimens and their susceptibility to antimicrobial agents are among the most important diagnostic measures of care for patients with cystic fibrosis (CF). Under the umbrella of EuroCareCF, two quality assurance trials of CF microbiology were performed in 2007 and 2008. Nine formulations with CF bacterial isolates were dispatched. A total of 31/37 laboratories from 18/21 European countries participated in the 2007 and 2008 trials. The common CF pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* were correctly identified by almost all participants in both trials, even if the strains presented uncommon phenotypes. *Burkholderia cenocepacia* IIIB and *Burkholderia vietnamensis* CF isolates, however, were correctly assigned to the species level by only 26% and 27% of the laboratories, respectively. Emerging pathogens such as *Achromobacter xylosoxidans*, *Inquilinus limosus*, and *Pandoraea pnomenusa* were also not detected or were misclassified by many laboratories. One participant correctly identified all CF isolates in both trials. The percentages of correct classifications (susceptible, intermediate, resistant) by antimicrobial susceptibility testing ranged from 55 to 100% (median, 96%) per isolate and drug. The shortcomings in the diagnostics of rare and emerging pathogens point to the need for continuing education in CF microbiology and suggest the establishment of CF microbiology reference laboratories.

The monogenic disorder cystic fibrosis (CF) predisposes individuals to chronic airway infections with opportunistic bacterial pathogens (16, 18, 19, 23). The bacteria most frequently isolated from the sputum of patients with CF by standard aerobic microbiological methods are *Staphylococcus aureus*, noncapsulated *Haemophilus influenzae*, and *Pseudomonas aeruginosa* (6). Individuals with CF are, moreover, susceptible to chronic respiratory tract infection with gram-negative bacterial species, which are intrinsically resistant to a broad range of antimicrobial agents and which are usually poor airway colonizers and not pathogenic for healthy persons (7). These rare and/or emerging pathogens in CF include *Stenotrophomonas maltophilia* (1); *Achromobacter* (*Alcaligenes*) *xylosoxidans* (15); *Inquilinus limosus* (20); and several species within the genera *Burkholderia* (12, 13), *Ralstonia* (12), and *Pandoraea* (11). Recent 16S rRNA gene profiling of CF respiratory secretions uncovered a further layer of complexity of CF microbiology (17). The bacterial community within the CF lung was found to be polymicrobial in nature and to include a range of anaerobic species primarily within the genera *Prevotella*, *Veillonella*, *Propionibacterium*, and *Actinomyces* (17, 21, 25).

A further characteristic feature of CF isolates that impedes the straightforward identification of taxa is their broad spectrum of numerous and often atypical phenotypes (4, 9, 14, 22). For example, a *P. aeruginosa* clone may diversify in CF lungs into different morphotypes (14), such as small-colony variants, alginate-overproducing mucoid variants, nonpigmented variants, or colonies with visible autolysis or autoaggregative behavior, all of which carry other adaptive mutations, metabolic features, and antimicrobial susceptibility patterns.

Present day CF microbiology services play a central role in the management of CF. Sensitive issues are the detection of transmissible pathogens, the emergence of multidrug-resistant variants, and the control of the efficacy of hygienic measures. To master these tasks, the clinical microbiology laboratory should have profound knowledge of the recent progress in the molecular taxonomy of CF pathogens, particularly among the betaproteobacteria, and the broad spectrum of uncommon phenotypes of isolates, particularly those from elderly CF patients (4). These demands are not trivial, and hence, the authors organized two quality assurance trials to address the issue of whether current knowledge in CF microbiology is translated into the microbiology services provided by the CF clinic. The trials asked for the species identification and antimicrobial susceptibilities of isolates from CF airways. Laboratories from 26 European countries which provide CF microbiology services for the largest CF centers in their home country were invited to participate. The trials identified shortcomings in the detection of rare and/or emerging pathogens which point to the need for continuing education in CF microbiology to promptly translate state-of-the-art knowledge into the daily practice of the clinical microbiology laboratory.

MATERIALS AND METHODS

Selection, characterization, and formulation of test strains. The test strains were subcultures of isolates that had been recovered from respiratory secretions

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TABLE 1. Quality assurance trials: species identification

Trial and strain or sample no.	Species	No. (% or organism) of participants providing the following response:			
		Correct	Incomplete	Wrong	Not detected
First trial (n = 31 participants)					
ECF-1	*B. cenocepacia* IIIB	3 (10)	5 (*B. cenocepacia*) 19 (*B. cepacia* complex)	4	
ECF-2	*A. xylosoxidans*	11 (35)		20	
ECF-3	*P. aeruginosa*	24 (77)		7	
ECF-Mix1	*P. aeruginosa*	31 (100)			
	S. aureus, SCV[b]	31 (100)			
ECF-Mix2	*P. aeruginosa*	29 (94)		2	
	A. xylosoxidans	19 (61)	4 (*Achromobacter* spp.)	7	1
	P. pnomenusa	7 (23)	4 (*Pandoraea* spp.)	14	6
Second trial (n = 37 participants)					
ECF-4	*B. vietnamensis*	10 (27)	16 (*B. cepacia* complex)	11	
ECF-5	*S. apiospermum*	31 (84)		6	
ECF-Mix3[a]	*S. aureus*	37 (100)			
	P. aeruginosa (a)	36 (97)		1	
	P. aeruginosa (b)	28 (76)		1	8
ECF-Mix4	*P. aeruginosa*	36 (97)		1	
	I. limosus	23 (62)		4	10

[a] *P. aeruginosa* clonal variants, designated *P. aeruginosa* (a) and *P. aeruginosa* (b), exhibited different MIC patterns, morphotypes, and growth behaviors.
[b] SCV, small-colony variant.

(bronchoalveolar lavage, sputum, and deep throat swab specimens) of individuals with CF who were regularly seen at the CF clinics in Munich or Hannover, Germany. The isolates had been stored as glycerol stock cultures at −80°C or for a maximum of 6 months on agar slants at ambient temperature. The strains were examined at the trial organizers' laboratories for their morphotypes, growth behaviors on plates, biochemical phenotypes (determined with the API 20NE and Vitek 2 systems), 16S rRNA gene sequences, and *recA* sequences for *Burkholderia* sp. (13). *Scedosporium apiospermum* was identified to the species level by sequencing of the internal transcribed spacer region (24).

Antimicrobial susceptibility was assessed at least in triplicate in Mueller-Hinton broth or on Mueller-Hinton agar for each drug by microdilution, agar dilution, and Etest (AB Biodisk, Solna, Sweden) (2, 3, 5, 8). Breakpoints were defined by use of the guidelines of the CLSI (5). If no values were available in the CLSI document, the classification of DIN 58940 (8) was followed. The MICs were translated into antimicrobial susceptibility categories (resistant, intermediate, susceptible) and were combined from the three methods for each drug. In the case of variable results (resistant and intermediate or susceptible and intermediate) both categories were accepted as correct. In the case of discrepant results for more than one category (resistant, intermediate, susceptible), the drug was excluded from analysis due to poor test performance, which was the case for the piperacillin susceptibility of *B. cenocepacia* strain ECF-1.

Only strains with reproducible and consistent phenotypes were selected for use in the trials. For formulation, strains were grown in Luria-Bertani broth at 37°C and the harvested biomass was lyophilized. The lots of lyophilized microbes were then checked for viability, culturability, and the original biochemical and antimicrobial susceptibility profiles.

Trial work flow. Clinical microbiology laboratories from 26 European countries were invited to participate in the two quality assurance trials of CF microbiology. Only laboratories which provided microbiology services for the largest CF clinics in their home country and/or which had a documented record of active participation at CF conferences and in peer-reviewed publications on CF microbiology during the last 5 years were contacted. Thirty-five of 53 invited laboratories agreed to participate in the first trial, and 40 of 61 invited laboratories agreed to participate in the second trial. Completed templates were submitted by 31 laboratories in the 2007 trial and by 37 laboratories in the 2008 trial. Twenty-nine laboratories took part in both trials. All contacted CF microbiology services from Belgium, The Netherlands, and the Scandinavian countries took part, whereas despite at least three electronic or written invitations and reminders, no laboratories from Estonia, Poland, the Czech Republic, Slovakia, and Bulgaria could be recruited for the first trial and no laboratories from Estonia, Latvia, Poland, and Hungary could be recruited for the second trial.

For each trial, formulations of single isolates and mixtures of two or three strains (Table 1) were dispatched by courier service. The participants were asked to identify the taxon of the recovered strains and to determine the antimicrobial susceptibility patterns of a subset of strains. An electronic template requested information about the genus and species of the strains recovered, the agar media used for cultivation, and the methods used for species identification. Boxes could be clicked to indicate whether automated systems were used or whether isolates were typically sent to another laboratory for further characterization. The participants were asked to indicate their antimicrobial susceptibility testing guidelines and antimicrobial susceptibility testing methods (agar dilution, microdilution, automated system/manufacturer; disc diffusion, Etest). Gram-positive bacteria were requested to be tested for their susceptibilities to penicillin G, oxacillin, gentamicin, vancomycin, and levofloxacin; and gram-negative bacteria to be tested for their susceptibilities to piperacillin, ceftazidime, meropenem, ciprofloxacin, levofloxacin, tobramycin, amikacin, trimethoprim-sulfamethoxazole, and colistin. Participants should preferentially determine the MIC (μg/ml). If this procedure was not at hand or inconvenient, the laboratories should report the zone diameter (mm) and disc content of the antimicrobial agent (μg) and provide a categorical assessment (susceptible, intermediate, or resistant). If CLSI methodology/breakpoints were not used, the laboratory was asked to fill in the breakpoints of their domestic guidelines.

RESULTS AND DISCUSSION

Nine formulations of single strains or mixtures were analyzed in the two quality assurance trials. The participants used 3 to 9 agar media (average, 5.6) for the recovery of the formulated strains. An agar selective for *Burkholderia cepacia* complex was included by all but three participants. Phenotypic tests for species identification were used by all laboratories. Automated systems were employed by 13 and 10 participants for the identification of common (*S. aureus*, *P. aeruginosa*) and uncommon pathogens, respectively. 28S rRNA and 16S rRNA gene sequencing capabilities were in place at 13 sites. Five laboratories each applied PCR tests to identify *P. aeruginosa* and the taxa of the *B. cepacia* complex.

Table 1 summarizes the outcome of species identification. Most test strains were selected from chronically infected CF airways in order to cover the habitat-specific features of bacterial diversification of morphotype, metabolic proficiency, or antimicrobial susceptibility. A small-colony variant (22) of *S. aureus* (ECF-Mix1) was correctly identified by all participants.

TABLE 2. Classifications by antimicrobial susceptibility quality assurance testing trials

Antimicrobial agent	% of the following isolates receiving the indicated classification[a]																	
	B. cenocepacia IIIB ECF-1			*A. xylosoxidans* ECF-2			*P. aeruginosa* ECF-3			*B. vietnamensis* ECF-4			*P. aeruginosa*					
													ECF-Mix3[b]			ECF-Mix3[b]		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Piperacillin	NR[c]			**86**	4	10	**100**			**66**	**17**	17	**96**	4% (I)				**100**
Ceftazidime	**94**	6			6	**94**	**97**	3		**79**	**12**	9	**100**			7	10	**83**
Meropenem	**63**	28	9	7	10	**83**	**97**	3		**76**	15	9	**94**	6		**100**		
Ciprofloxacin	3		**97**	23	**45**	**32**	**36**	**27**	37	**65**	**27**	8	**93**	7		**57**	**35**	8
Levofloxacin	6		**94**	**83**	**17**		**33**	**22**	45	**63**	26	12	**95**	5		19	**31**	**50**
Tobramycin			**100**			**100**	36	**27**	**36**	17	7	**76**	**100**			**100**		
Amikacin			**100**	3		**97**	3	**7**	**90**	16	3	**81**	**90**	10		**92**	4	4
TMP-SMX[d]	7	3	**90**	25		**75**			**100**	21	10	**69**	13		**87**			**100**
Colistin			**100**			**100**	**97**		3	3		**97**	**96**	4		**100**		

[a] Strains were classified as susceptible (S), intermediate (I), or resistant (R) to each antimicrobial agent according to the guidelines provided by the CLSI (5). If no values were available in the CLSI document, the classification by DIN 58940 (8) was followed. If CLSI methodology/breakpoints were not used by the respective laboratory, the raw quantitative data were converted into CLSI susceptible, intermediate, or resistant categories. The correct categorization is indicated in boldface.

[b] ECF-Mix3 also contained an *S. aureus* isolate. All participants correctly indicated that the strain was susceptible to penicillin G, oxacillin, gentamicin, vancomycin, and levofloxacin.

[c] NR, not reproducible.

[d] TMP-SMA, trimethoprim-sulfamethoxazole.

However, about a quarter of the laboratories failed to differentiate two clonal variants of *P. aeruginosa* with divergent growth and antimicrobial susceptibility profiles (ECF-Mix3). A *P. aeruginosa* strain with an uncommon metabolic profile (strain ECF-3) (4) was also not recognized by a substantial minority of participants. Overall, however, the most common pathogens, *S. aureus* and *P. aeruginosa*, were correctly identified by most laboratories (Table 1).

The results were quite different for less common or emerging pathogens. Infections caused by members of the *B. cepacia* complex present a hazard for the CF population because of patient-to-patient transmission and the potentially fatal outcome (10, 19). Since the taxa of the genus are endowed with different pathogenicities (10, 12, 19), knowledge of the species is important for the management of the infected CF patient. Correspondingly, the template requested identification of the organisms to the species level. Half of the CF microbiology laboratories, however, stopped the analysis with the determination "*B. cepacia* complex." Thus, these laboratories did not differentiate between the harmful *B. cenocepacia* IIIB isolate (strain ECF-1) (12) that had caused severe pulmonary exacerbations in the affected patient and a *B. vietnamensis* strain (strain ECF-4) with a low level of pathogenicity (12) that had chronically colonized the airways of a CF adult without any clinical signs of infection. The laboratories which differentiated the *Burkholderia* spp. to the species level had incorporated 16S rRNA gene sequencing and further molecular techniques, such as *recA* sequencing and in-house PCR tests, into their clinical routines. At a few other sites which followed their common regimen to send samples to a sequencing service upon demand, the results were reported after the deadline of the quality assurance trial. Six of the 15 laboratories that performed DNA sequencing in-house indicated that they typically send *B. cepacia* complex isolates to another laboratory for refined taxonomic assignment.

The highest rate of misclassification was observed for emerging pathogens. The correct detection of *I. limosus* (20), an atypical *A. xylosoxidans* isolate, and *Pandoraea pnomenusa* was made by 62%, 35%, and 23% of participants, respectively. *I. limosus* was not detected in the formulated strain mixture by 10 laboratories or was misclassified as *Brevundimonas vesicularis* (once), *Sphingomonas paucimobilis* (once), non-lactose fermenter (once), or a member of the *Burkholderia cepacia* complex (once). The incorrect species identifications of the *A. xylosoxydans* strain included gram-negative organism (10 times), *Ochrobactrum anthropi* (once), *Comamonas* sp. (once), suspected *Achromobacter* (2 times), *Achromobacter denitrificans* (3 times), *Pseudomonas alcaligenes* (once), *Pseudomonas* sp. (once), and *Corynebacterium* sp. (once). The *P. pnomenusa* isolate in ECF-Mix 2 was not detected by six participants or was classified as a gram-negative organism (10 times), *Pandoraea* sp. (4 times), *B. cepacia* (once), *Burkholderia* sp. (once), *Pseudomonas* sp. (once), or *Wautersia paucula* (once).

Better performance was noted for antimicrobial susceptibility testing (Table 2). The majority of laboratories did not test all antimicrobial agents listed in the template. All laboratories included ceftazidime, meropenem, ciprofloxacin, and at least one aminoglycoside in their antimicrobial susceptibility tests. Levofloxacin was the drug that was the least tested, being tested by just 60% of participants. Hence, the data on susceptible, intermediate, and resistant categories submitted were normalized (Table 2). The majority of laboratories applied disc diffusion and/or Etest. A microdilution method was applied by four laboratories. The antimicrobial susceptibility of *S. aureus* was determined at eight sites by automated systems, but for the other taxa, automated systems were employed by only three laboratories. In general, the participants made only minor mistakes. The median frequency of incorrect categories was 4% (range, 0 to 45%; Table 2). A major outlier was the atypical *P. aeruginosa* ECF-3 isolate, for which close-to-random assessments of its susceptibility to ciprofloxacin, levofloxacin, and tobramycin were made. Moreover, the tests with sulfonamides and quinolones often revealed erroneous results (Table 2).

The laboratories were ranked by the number of mistakes. Eight laboratories from Belgium, Germany, Italy, The Netherlands, and Sweden correctly identified all strains and made no major mistakes in antimicrobial susceptibility testing for more than one category in at least one trial. One of these eight participants correctly identified all CF isolates in both trials.

There was no association between the ranking of a laboratory within the quality assurance trial and the research activities of its institution on CF microbiology, as assessed by the number of publications during the last 5 years as a rough surrogate parameter. The quartile with the largest number of mistakes was populated by laboratories located in southern, southeastern, or eastern European countries. Thus, the outcome of the trials points to a gradient of the quality of CF microbiology services in Europe that is apparently not influenced by a local research focus on CF but, rather, that is determined by the available resources and by domestic clinical microbiology quality control guidelines.

Considering that the participants provide clinical microbiology services for the largest and/or most renowned CF centers in their home country and that the majority pursue CF research, the quality assurance trials uncovered unexpected deficits that would probably have been even more dramatic if an unselected cohort of clinical microbiology laboratories had been recruited for the trials.

The following shortcomings were noted. First, *P. aeruginosa* strains that exhibited an atypical phenotypic signature (4, 9, 14, 22), such as slow growth, auxotrophy, or uncommon morphotypes, seemed to be difficult to be recognized, even by the expert in CF microbiology. Second, most participants had thorough knowledge of the molecular taxonomy of the genus *Burkholderia* (12), but this knowledge was not translated into the microbiology routine. Just 16 laboratories had 16S rRNA sequencing as a routine in place. Third, rare emerging pathogens (7) turned out to be a major challenge for the CF microbiology experts. Finally, *S. apiospermum*, a filamentous fungus with the potential to cause invasive infections, especially in lung transplant patients, was correctly identified by just 84% of the participants. Due to technical constraints, we did not include nontuberculous mycobacteria or any anaerobic organism that, according to recent studies on the bacterial metagenome (17, 21), represent a major portion of the polymicrobial communities in CF lungs. Although the clinical relevance of anaerobic bacteria for CF lung infection is an open question, we can envisage that CF microbiology will soon become much more complex for the routine clinical microbiology than it is now. Further external quality assessments, continuing education in CF microbiology, and the establishment of CF microbiology reference laboratories should allow the shortcomings identified in these quality assessment trials to be reduced and allow the foreseeable challenges of the CF microbiology of emerging pathogens, novel phenotypes, and bacterial metagenomics to be coped with in the future.

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