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.

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.
Germany. The isolates had been stored as glycerol stock cultures at
with CF who were regularly seen at the CF clinics in Munich or Hannover,
(bronchoalveolar lavage, sputum, and deep throat swab specimens) of individuals

TABLE 1. Quality assurance trials: species identification

<table>
<thead>
<tr>
<th>Trial and strain or sample no.</th>
<th>Species</th>
<th>No. (% or organism) of participants providing the following response:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Correct</td>
</tr>
<tr>
<td>First trial (n = 31 participants)</td>
<td>B. cenocepacia IIIB</td>
<td>3 (10)</td>
</tr>
<tr>
<td>ECF-2</td>
<td>A. xylosoxidans</td>
<td>11 (35)</td>
</tr>
<tr>
<td>ECF-3</td>
<td>P. aeruginosa</td>
<td>24 (77)</td>
</tr>
<tr>
<td>ECF-Mix1</td>
<td>P. aeruginosa</td>
<td>31 (100)</td>
</tr>
<tr>
<td>ECF-Mix2</td>
<td>S. aureus, SCVb</td>
<td>31 (100)</td>
</tr>
<tr>
<td>ECF-Mix3</td>
<td>P. aeruginosa</td>
<td>29 (94)</td>
</tr>
<tr>
<td></td>
<td>A. xylosoxidans</td>
<td>19 (61)</td>
</tr>
<tr>
<td></td>
<td>P. pseudomurea</td>
<td>7 (23)</td>
</tr>
<tr>
<td>Second trial (n = 37 participants)</td>
<td>B. vietnamensis</td>
<td>10 (27)</td>
</tr>
<tr>
<td>ECF-4</td>
<td>S. apiosepermum</td>
<td>31 (84)</td>
</tr>
<tr>
<td>ECF-5</td>
<td>S. aureus</td>
<td>37 (100)</td>
</tr>
<tr>
<td>ECF-Mix3a</td>
<td>P. aeruginosa (a)</td>
<td>36 (97)</td>
</tr>
<tr>
<td></td>
<td>P. aeruginosa (b)</td>
<td>28 (76)</td>
</tr>
<tr>
<td>ECF-Mix4</td>
<td>P. aeruginosa</td>
<td>36 (97)</td>
</tr>
<tr>
<td></td>
<td>I. limosus</td>
<td>23 (62)</td>
</tr>
</tbody>
</table>

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.

(condrochalveolar 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
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

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, cultureability, and the original biochemical and anti-
microbial susceptibility profiles.

Trial work flow. Clinical microbiology laboratories from 26 European coun-
tries were invited to participate in the two quality assurance trials of CF micro-
biology. Only laboratories which provided microbiology services for the largest
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 par-
ticipants were asked to indicate their antimicrobial susceptibility testing guide-
lines and antimicrobial susceptibility testing methods (agar dilution, microdilu-
tion, 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-sulfamethox-
azole, 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 ana-
yzed in the two quality assurance trials. The participants used
3 to 9 agar media (average, 5.6) for the recovery of the for-
multrated 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 bac-
terial diversification of morphotype, metabolic proficiency, or
antimicrobial susceptibility. A small-colony variant (22) of S.
aureus (ECF-Mix1) was correctly identified by all participants.
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. vietnamiensis* 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. xylosoxidans* 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 cefazidime, meropenem, ciprofloxacin, and at least one aminoglycoside in their antimicrobial susceptibility tests. Levofloxacin was the drug that was the least tested, being included by just 60% of participants. Hence, the data on susceptibility testing (Table 2). The majority of laboratories did not test all antimicrobial agents listed in the template. All laboratories included cefazidime, meropenem, ciprofloxacin, and at least one aminoglycoside in their antimicrobial susceptibility tests. Levofloxacin was the drug that was the least tested, being included by just 60% of participants. Hence, the data on susceptibility, 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.

### TABLE 2. Classifications by antimicrobial susceptibility quality assurance testing trials

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>B. cenocepacia IIIB</th>
<th>A. xylosoxidans</th>
<th>P. aeruginosa</th>
<th>B. vietnamiensis</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ECF-1</td>
<td>ECF-2</td>
<td>ECF-3</td>
<td>ECF-4</td>
<td>ECF-Mix3b</td>
</tr>
<tr>
<td>Piperacillin</td>
<td></td>
<td>S I R</td>
<td>S I R</td>
<td>S I R</td>
<td>S I R</td>
</tr>
<tr>
<td>NR</td>
<td>94</td>
<td>6</td>
<td>94</td>
<td>6</td>
<td>94</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>6</td>
<td>94</td>
<td>6</td>
<td>93</td>
<td>94</td>
</tr>
<tr>
<td>Meropenem</td>
<td>63</td>
<td>28</td>
<td>9</td>
<td>10</td>
<td>44</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>3</td>
<td>97</td>
<td>23</td>
<td>45</td>
<td>94</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>6</td>
<td>94</td>
<td>83</td>
<td>17</td>
<td>95</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>93</td>
</tr>
<tr>
<td>Amikacin</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>93</td>
</tr>
<tr>
<td>TMP-SMX</td>
<td>7</td>
<td>3</td>
<td>90</td>
<td>25</td>
<td>93</td>
</tr>
<tr>
<td>Colistin</td>
<td>100</td>
<td>100</td>
<td>97</td>
<td>75</td>
<td>93</td>
</tr>
</tbody>
</table>

* 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.
* 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.
* NR, not reproducible.
* TMP-SMA, trimethoprim-sulfamethoxazole.
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 providers conduct 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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REFERENCES


