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Effect of Media Modified To Mimic Cystic Fibrosis Sputum on the Susceptibility of *Aspergillus fumigatus*, and the Frequency of Resistance at One Center

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Studies of cystic fibrosis (CF) patient exacerbations attributed to *Pseudomonas aeruginosa* infection have indicated a lack of correlation of outcome with *in vitro* susceptibility results. One explanation is that the media used for testing do not mimic the airway milieu, resulting in incorrect conclusions. Therefore, media have been devised to mimic CF sputum. *Aspergillus fumigatus* is the leading fungal pathogen in CF, and susceptibility testing is also used to decide therapeutic choices. We assessed whether media designed to mimic CF sputa would give different fungal susceptibility results than those of classical methods, assaying voriconazole, the most utilized anti-*Aspergillus* drug in this setting, and 30 CF *Aspergillus* isolates. The frequency of marked resistance (defined as an MIC of >4 μg/ml) in our CF unit by classical methods is 7%. Studies performed with classical methods and with digested sputum medium, synthetic sputum medium, and artificial sputum medium revealed prominent differences in *Aspergillus* susceptibility results, as well as growth rate, with each medium. Clinical correlative studies are required to determine which results are most useful in predicting outcome. Comparison of MICs with non-CF isolates also indicated the CF isolates were generally more resistant.

Cystic fibrosis (CF) is a genetic disorder in epithelial anion transport. One result is the development of thick respiratory secretions, which are associated with repeated pulmonary infections and inflammation. The most common bacterium associated with these infections is *Pseudomonas aeruginosa*. The most common fungal pathogen associated with CF airways is *Aspergillus fumigatus*. Infection with either has been associated with worsened or a more rapid decline in CF pulmonary function (1–11), with coinfected patients having the worst prognosis (9, 12). In addition, *Aspergillus fumigatus* causes an allergic bronchopulmonary disease in up to 15% of CF patients (13).

An important observation made with respect to *Pseudomonas aeruginosa* infection in CF patients is the lack of correlation of classical *in vitro* susceptibility testing of isolates from patients with exacerbations with the treatment outcomes in such patients (14, 15). One logical explanation for that dissociation is that classical *in vitro* susceptibility testing does not mimic the conditions present in CF airways, thus leading to erroneous conclusions with respect to the activity of various antibacterials. For that reason, various researchers have attempted to devise media for use in the laboratory that more closely approximate the milieu of CF airways for purposes of susceptibility testing, as well as studying growth, physiology, mutability, etc., of *P. aeruginosa* under such conditions. Such media have been shown to give disparate results from those obtained for *P. aeruginosa* using classical methods (16), such as the methods and medium of the Clinical and Laboratory Standards Institute.

With standardization of susceptibility testing for fungi, we wondered whether the routine *in vitro* conditions for testing *A. fumigatus*, and on which therapeutic antifungal decisions in CF are based, might also give divergent results from media constituted to more closely approximate CF airway contents and sputum. We also compared, using classical *in vitro* susceptibility testing, the MIC results obtained with CF and with non-CF isolates.

MATERIALS AND METHODS

Isolates. CF isolates from patient respiratory cultures were obtained after written informed consent for biobanking of the patients' specimens and subsequent use. This collection was approved by the Stanford University Institutional Review Board. Thirty *A. fumigatus* isolates from 28 different CF patients were studied; one patient provided 3 different isolates at different times. All were identified as *A. fumigatus sensu stricto* (17).

Non-CF isolate susceptibility data were from requested testing in our laboratory of clinical isolates from patients with invasive or suspected invasive aspergillosis.

Media. The three media described in the literature were prepared. All were prepared under sterile conditions and were stored in the dark at 4°C when not in use. The pH of all three media ranged from 6.8 to 7.0.

(i) Digested sputum medium. Digested sputum medium was originally described by Davis and Bruns (18) as an equal mixture of pooled, ultrasonically homogenized, autoclaved CF sputum and broth. A later modification (16) used dithiothreitol-digested sputum. We obtained 10 fungus culture-negative sputum samples from CF outpatients in 0.3- to 0.5-ml amounts and pooled them. The sputum samples were obtained after written informed consent for biobanking of the patients' specimens and subsequent use, and this was approved by the Stanford Institutional Review Board. Concordant with methods previously described (19, 20), the pool was mixed with an equal volume of 0.1% dithiothreitol (Calbiochem, San Diego, CA) in normal saline, filtered through a 0.2-μm filter,

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vortex-mixed for 3 s, and incubated for 5 min in a 37°C water bath with vigorous shaking. Further mixing was performed by aspirating up and down 20 times in a pipette. The incubations and pipetting were repeated 2 more times. Any residual unsolubilized material was removed by centrifugation at 800 × *g* for 5 min at 4°C. The supernatant was then tested as 50:50, 25:75, and 10:90 mixtures with RPMI 1640 medium and a reference *A. fumigatus* isolate, strain 10AF (21), to determine which mixture gave optimal *A. fumigatus* growth at 37°C. The 10:90 mixture with RPMI 1640 was then selected for further studies. A control medium was prepared with equal volumes of RPMI 1640 (in lieu of sputum) and 0.1% dithiothreitol and was then subjected to all of the steps previously described. This dithiothreitol control medium thus had a final concentration of 0.005% dithiothreitol, which is the same as the test medium.

(ii) Synthetic sputum medium. In contrast to the digested sputum medium, Palmer et al. (22) developed a defined medium, based on CF sputum analyses, that nutritionally mimics CF sputum. The amino acids and carbon sources are supplemented with 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer to mimic the native buffering system of the lung, and we followed the recipe detailed elsewhere (22). MOPS is the same buffer as that in the standard RPMI test medium used.

(iii) Artificial sputum medium. Sriramulu et al. developed a partially defined medium with a composition that closely resembles CF sputum (23). Notable in the contents, aside from the amino acids, are mucin, DNA, Tris buffer, and egg yolk emulsion (source of lecithin). We prepared a filtered (0.2 μm) version of this medium as detailed by Kirchner et al. (24) using herring sperm as the source of the DNA (Sigma, St. Louis, MO) and egg yolk emulsion (Fisher Scientific, Hampton, NH) as the source of the lecithin.

Testing. For testing with each of the three media described, MICs of three different sets of 10 randomly selected CF sputum *A. fumigatus* isolates were determined by a classical broth macrodilution method as detailed (25). Each of the 3 test media was tested with a different set of the 10 isolates concurrently with the standard RPMI 1640 medium described (25). The 3 isolates from a single patient were randomized to 2 different sets of 10. Minimum fungicidal concentrations (MFCs) were determined as previously described (26), with the cutoff representing ≥96% killing of the initial inoculum. Voriconazole was selected as the test drug, as it is the most recommended for therapy against aspergillosis (27), it is most used in the Stanford CF clinic, and because differences in susceptibility among various isolates are greater with azoles than with polyenes (28). Isolates appearing resistant (≥4 μg/ml) had their tests repeated. The non-CF isolates had MIC testing performed by the same method, but requests for MFC testing were sporadic. In every run, a known voriconazole-susceptible isolate (*Candida kefyr*, isolate SA) was tested concurrently in the standard medium to ensure drug potency. In standard medium and in synthetic sputum medium, the isolates grew to 4+ in the absence of drug in 2 days (the time of reading for them); in digested sputum medium, it required 5 days under the culture conditions (25) to obtain 4+ growth in the absence of drug (the time of reading).

Statistical analysis. In all tests, a P value of <0.05 was considered significant. For analysis of the CF isolates studied in test medium compared with those studied under standard conditions, done in 2-fold dilutions, the MIC data were transformed to log base 2, with MIC and MFC values of ≤0.5 and >8 μg/ml scored as 0.5 and 16, respectively. A comparison was then done using the Wilcoxon paired signed-rank test (29). For comparison of the CF and non-CF groups, a contingency chi-square test was performed to test for differences in the distribution of the MIC values (values of 8 and >8 were combined as ≥8 μg/ml for this analysis). After demonstration of a significant distribution difference, a Fisher exact test was performed at each MIC value.

RESULTS

Digested sputum medium. The results with the digested sputum medium, control medium, and standard medium are shown in Table 1. In the absence of drug, the fungal growth was noted to clump in standard medium or dithiothreitol control medium, but it grew as dispersed tiny balls in digested sputum medium. One of the 10 isolates (number 4) was completely resistant (MIC and MFC value of >8 μg/ml) to voriconazole in standard testing. Dithiothreitol alone did not appear to affect the overall susceptibility profile (dithiothreitol control medium), but one isolate (number 9) had a minimal 2-fold increase in MIC and MFC.

TABLE 1 Results with digested sputum medium

Medium and MIC or MFC	No. of isolates with the following result (in μg/ml):					
	≤0.5	1	2	4	8	>8
Standard (RPMI)						
MIC	1	3	2	3[a]	0	1[b]
MFC	1	3	2	3[a]	0	1[b]
Dithiothreitol control medium						
MIC	1	2	2	3	1[a]	1[b]
MFC	1	2	2	3	1[a]	1[b]
Digested sputum medium						
MIC	4	3	3[a,b]	0	0	0
MFC	1	0	0	2	2	5[a,b]

[a] This group includes isolate no. 9.
[b] This group includes isolate no. 4.

Remarkably and unexpectedly, the digested sputum medium resulted in a shift of the set toward greater susceptibility to inhibition (MIC) and toward greater resistance to killing (MFC). The relative positions of the isolates in terms of susceptibility within the group appeared not to change, and the 2 most resistant isolates (number 4 and number 9) were always at the resistant end of the spectrum regardless of the medium used. However, in the digested sputum medium, the otherwise consistently resistant isolate (number 4) would have been assigned an MIC (2 μg/ml) that could be exceeded in serum with usual dosing regimens. Three isolates had unchanged MICs in digested sputum medium, but the other 7 decreased 1 to 3 tube dilutions ($P = 0.009$). Two had unchanged MFCs, but the other 8 increased 1 to 4 tube dilutions ($P = 0.006$).

Synthetic sputum medium. The results with the synthetic sputum medium and the standard medium are shown in Table 2. No unusual features of growth in the absence of drug were noted. One of 10 isolates (number 19) was completely resistant (MIC and MFC values of >8 μg/ml) to voriconazole in standard testing. Remarkably, the synthetic sputum medium also resulted in a downshift of the set toward greater susceptibility (MIC and MFC) compared to that of the standard medium. Of note, the most resistant isolate (number 19) was resistant in both media.

Three isolates had an unchanged MIC in synthetic sputum medium, but the other 7 decreased 1 or 2 tube dilutions ($P = 0.009$). Three isolates had an unchanged MFC in synthetic sputum medium, but the other 7 decreased 1 or 2 tube dilutions ($P = 0.009$).

Artificial sputum medium. In the standard medium, all 10 isolates had MICs and MFCs of ≤4 μg/ml (Table 3, third cohort). However, all isolates failed to grow sufficiently in artificial sputum medium.

Overall susceptibility of *A. fumigatus* in the Stanford CF population and comparison to the non-CF population. Summa-

TABLE 2 Results with synthetic sputum medium

Medium and MIC or MFC	No. of isolates with the following result (in μg/ml):					
	≤0.5	1	2	4	8	>8
Standard (RPMI)						
MIC	1	4	4	0	0	1[a]
MFC	1	4	4	0	0	1[a]
Synthetic sputum medium						
MIC	8	1	0	0	0	1[a]
MFC	8	1	0	0	0	1[a]

[a] This group includes isolate no. 19.

TABLE 3 Results of testing the third CF cohort and non-CF isolates in standard medium

Cohort, medium, and MIC or MFC	No. of isolates with the following result (in μg/ml):					
	≤0.5	1	2	4	8	>8
Third CF cohort, standard (RPMI)[a]						
MIC	0	0	2	8	0	0
MFC	0	0	1	9	0	0
All CF isolates, standard (RPMI)						
MIC	2	7	8	11	0	2
All non-CF isolates, standard (RPMI)						
MIC	23	10	16	4	1	1

[a] The third cohort of isolates was tested concurrently with standard medium and artificial sputum medium but failed to grow sufficiently in the latter to yield endpoints.

rizing the above results, 2 of 30 (7%) isolates were in the highly resistant (MIC, >4 μg/ml) category with the standard medium and method (Table 3, top). Neither of these 2 resistant isolates came from a patient who had received antifungals previously.

Compared with the non-CF isolates with the same standard medium and method, the CF isolates were more resistant (MIC) ($P = 0.001$) (Table 3, bottom). This was because of differences at the ≤0.5 μg/ml MIC ($P < 0.0001$) and at the 4 μg/ml MIC ($P = 0.02$). The frequency of highly resistant isolates, though almost twice as common in the CF group (2/30) than in the non-CF group (2/55), was not statistically significant.

The 3 isolates from one patient produced 2 different susceptibility patterns. Four of the 28 CF patients had allergic bronchopulmonary aspergillosis, but only one had received an azole prior to the isolate we obtained. Of the other 24 CF patients, only 2 had received an azole (voriconazole) prior to isolate collection.

DISCUSSION

To our knowledge, this is the first study of the effect of media mimicking CF sputum on *A. fumigatus* susceptibility results. We had the opportunity to test this with *A. fumigatus* isolates from CF patients. It may prove to be the case that tests using such designed media prove more relevant to clinical outcome than those using standard methods, but this will require correlations of *in vitro* results, clinical courses, and posttreatment cultures. This has not yet even been done with *P. aeruginosa* infections, although studies of growth and metabolism of that bacterium in these media have been performed (30, 31). Of note, the two media tested in which *A. fumigatus* could grow appeared to make the isolates generally more susceptible to drug inhibition, although digested sputum medium made the isolates more resistant to killing. It will be important to discover what components of the sputum or artificial media result in altered growth characteristics for the fungus and in altered drug susceptibility. The explanation does not lie in pH, in dithiothreitol (our studies comparing medium with and without dithiothreitol), or in any effect from saliva that may contaminate sputum samples, as Davis and Bruns (18) did not find saliva to affect test results. It is also of interest to know why one medium, in which *P. aeruginosa* grows adequately (31), inhibits growth of *A. fumigatus*. It is tempting to speculate that the principal inhibitor of fungi in artificial sputum medium is mucin (32) given that *P. aeruginosa* can use mucin as an energy source (23) and that mucin would be at least partially digested in digested sputum medium (in which *A. fumigatus* grows, albeit more slowly) by dithiothreitol but present in artificial sputum medium. Finally, it should also be realized that even the most artfully constructed medium to mimic CF sputum does not completely reconstruct the *in vivo* condition, where, e.g., partial oxygen pressure (pO_2), ionic composition, consistency, effects from other resident microbes, etc., may be different than those under *in vitro* conditions.

Of note, media using digested CF sputum have been tested for their effect on *P. aeruginosa* susceptibility *in vitro*. Serisier et al. (16) found such a medium to increase the susceptibility of *P. aeruginosa* to antibacterial inhibition, which is the same effect that we found with an antifungal. Davis and Bruns (18) found that the minimum bactericidal concentration increased for some antibacterials, which is remarkably also the same effect that we found with an antifungal; however, there was no change in killing with some other antibacterials (18). Synthetic sputum medium apparently has not been tested in *P. aeruginosa* susceptibility. Artificial sputum medium is reported to decrease the susceptibility of *P. aeruginosa* to antibacterials, but we cannot compare our results because *A. fumigatus* fails to grow sufficiently in that medium. Waters and Ratjen (15) concluded from the literature that results of susceptibility testing *P. aeruginosa* as a biofilm, regardless of medium, did not correlate any better with clinical outcome than did testing planktonic *P. aeruginosa*; whether this would apply to *A. fumigatus* biofilm (33) with these media is unknown.

With the frequency of *A. fumigatus* infections in CF patients and the use of antifungals in some for treatment, one might expect *A. fumigatus* drug resistance to possibly be a problem. In addition to the development of resistance during therapy, there is also the possibility of cross-infection with resistant *A. fumigatus* in the clinic setting (34). At this time, there is rising azole resistance of *A. fumigatus* in areas of Europe, with unique mutations in the azole fungal target attributed to concomitant use of azole antifungals in agricultural practice (35). Thus far, North America appears to rarely show this pattern (36). There are a few studies of resistance in *A. fumigatus*, specifically in CF patients from Europe. Some have reported CF azole resistance with a frequency similar to ours (34, 37, 38), whereas others have reported a very low frequency (39–41); this will vary with the definition of resistance and with the azole used to define resistance. Our resistance appears unrelated to prior antifungal use, as our 2 resistant isolates came from patients who had no prior antifungal therapy. This finding of resistance in the absence of drug exposure and the different patterns in one patient with multiple isolates in the absence of drug exposure are consistent with findings that *A. fumigatus* isolation from

CF sputum may be transient in many patients (34, 42–44). They may be infected transiently with a resistant isolate from the environment or from another patient (34). It has also been demonstrated that multiple *A. fumigatus* genotypes may at times coexist in the same patient (45, 46). The *cyp51A* gene of our 2 resistant isolates was sequenced, and none of the described nucleic acid sequence variants associated with azole resistance (47) (and presumed largely related to prior azole exposure) (35, 48) were found (D. W. Denning, personal communication). This implies a resistance based on drug efflux differences or mutations in other parts of the azole susceptibility pathways.

Overall, it was also notable that MICs of CF isolates in standard medium were generally and significantly higher than those of non-CF isolates. This has also been suggested previously (49). The frequency of marked resistance (MIC, ≥8 μg/ml) was, however, not significantly different in the two groups (2/30 versus 2/55). We were unable to compare prior antifungal experience in our CF and non-CF patients, as the latter was largely unknown. Some laboratories have recommended an MIC of 4 μg/ml as also resistant for voriconazole (50); this would have accentuated our conclusions about resistance frequency in CF. Testing with only voriconazole may have caused us to miss some isolates resistant to other azoles (other laboratories have used itraconazole to screen for azole resistance), as only some azole-resistant isolates are cross-resistant to all azoles.

In summary, with the shift toward resistance in our CF population and with the lack of evidence that this shift is related to azole drug pressures in the population, another hypothesis is suggested. Our hypothesis is that residence in the unusual milieu of the CF airways may alter drug penetration, azole target, or efflux, predisposing *A. fumigatus* to the small but highly significant population shift in voriconazole susceptibility.

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