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Bacteriology of Sputum in Cystic Fibrosis: Evaluation of Dithiothreitol as a Mucolytic Agent

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Liquefaction and homogenization have been recommended to ensure accurate, representative sputum cultures. We evaluated dithiothreitol (DTT) as a mucolytic agent for culturing sputum samples obtained from 79 cystic fibrosis (CF) patients. Liquefaction with DTT was not superior to direct plating of specimens for routine, qualitative cultures. Unliquefied sputum cultures failed to detect 3 of 47 *Pseudomonas aeruginosa* isolates; DTT-treated specimens missed 5 of 13 *Candida albicans* isolates. Neither treated nor untreated sputum cultures were completely successful in detecting *Staphylococcus aureus* or *Enterobacteriaceae*. Since *Haemophilus influenzae* was recovered from only two qualitative cultures, we could not evaluate the effect of DTT on the recovery of this organism. However, 27 of 29 strains of *H. influenzae* were inhibited by concentrations of DTT near the recommended final working concentration of 50 μg/ml, suggesting that liquefaction might impair isolation of this organism. Liquefaction with DTT permitted quantitative cultures of CF sputum. The predominant pathogen in our CF population was *P. aeruginosa*; 37 of 43 (86%) patients were colonized with this organism. Median densities of rough and mucoid strains were 3.2×10^7 and 4.3×10^7 colony-forming units per ml, respectively. Previous oral antistaphylococcal therapy may have accounted for the observed low density of *S. aureus* (mean density, 3.5×10^3 colony-forming units per ml). We conclude that DTT treatment does not improve recovery of organisms from qualitative cultures but does facilitate quantitative studies of *S. aureus* and *P. aeruginosa* in CF sputum.

Over 25 years ago, May (13) demonstrated that bacteria are not uniformly distributed in sputum. He suggested that accurate, representative cultures could only be obtained by sampling at least five separate parts of a specimen or by homogenizing the sputum. Since May's observations, a variety of mucolytic agents, including pancreatin (4, 19), pancreatin-trypsin (20), amylase (A. Balows, W. W. Allen, and P. E. Greenup, Bacteriol. Proc., p. 107, 1969), sodium 2-ethylhexyl sulfate (Tergemist; 6), and *N*-acetylcysteine (NAC, Mucomyst; 15, 18) have been used to liquefy sputum before homogenization. However, the usefulness of these agents in processing sputum from cystic fibrosis (CF) patients has not been adequately studied. Moreover, NAC, which is one of the more popular liquefying agents, has appreciable in vitro activity against *Pseudomonas aeruginosa*, the principal pathogen in the sputum of most CF patients (16). We therefore chose to study another mucolytic agent, dithiothreitol (DTT, Sputolysin), which is superior to other compounds in liquefying sputum (5). We examined the in vitro activity of DTT against bacteria frequently found in the sputum of CF patients, and we compared the recovery of bacteria from qualitative cultures liquefied with DTT and from specimens processed by standard methods. We also evaluated the potential usefulness of DTT in studying quantitative aspects of CF sputum microbiology.

MATERIALS AND METHODS

Preparation of DTT. Sputolysin (Calbiochem, La Jolla, Calif.) is a sterile solution of DTT packaged at 6.5×10^{-3} mol/liter (1 mg/ml) in pH 7 sodium phosphate buffer. Following the manufacturer's recommendations, sputolysin was diluted 10-fold in sterile distilled water to give a concentration of 100 μg of DTT per ml. This solution was maintained in an ice bath until used and was made fresh each day. The DTT solution was combined with an equal volume of sputum to yield a final concentration of 50 μg/ml.

Bacterial strains. The microorganisms studied were all isolated from clinical specimens of patients admitted to Children's Hospital Medical Center and were identified by standard laboratory methods (7). The 21 strains of *P. aeruginosa* and the 10 strains of *Staphylococcus aureus* were maintained on nutrient agar slants at room temperature, the four strains of *Candida albicans* were maintained on Sabouraud

dextrose agar at 4°C, and the 29 strains of *Haemophilus influenzae* were kept frozen at −70°C in sterile skim milk.

Antimicrobial activity of DTT. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined by a microdilution technique (10, 22). The following broths were used: Mueller-Hinton broth for *P. aeruginosa* and *S. aureus*, brain heart infusion (BHI) broth for *C. albicans*, and BHI broth supplemented with horse blood lysate and nicotinamide adenine dinucleotide (1) for *H. influenzae*. One-tenth-milliliter broths containing DTT concentrations of 0, 8, 16, 31.3, 62.5, 125, 250, and 500 μg/ml were inoculated with 10^4 colony-forming units (CFU) of the test organism in the logarithmic phase of growth. The MBC was defined as growth of less than five colonies when 0.01 ml of the test broth was subcultured on agar plates. The MIC and MBC were determined after incubation at 37°C for 24 and 48 h, respectively.

Effect of DTT on growth of test strains. Growth curves for selected strains of *H. influenzae* type b, *S. aureus*, *P. aeruginosa*, and *C. albicans* were performed to confirm the results of MIC and MBC testing and to determine whether the working DTT concentration had an early effect on growth not evident after 24 h of incubation. Growth curves were performed in appropriate broths as noted above. Ten-milliliter broths containing DTT at concentrations of 0, 0.5, 5, and 50 μg/ml were inoculated with 10^5 CFU of the test strains in the logarithmic phase of growth and were incubated at 37°C in a rotary shaker. At 0, 2, and 4 h, 0.1 ml of appropriate broth dilutions was inoculated onto Mueller-Hinton, BHI, or supplemented BHI plates. The plates were incubated at 37°C for 24 h, and the number of CFU was recorded.

Quantitative and qualitative sputum cultures. Sputum was obtained from a total of 79 patients with CF who were either hospitalized or seen in the outpatient clinics at Children's Hospital Medical Center. Qualitative sputum cultures, processed both with and without DTT liquefaction, were obtained from 36 inpatients. Sputum for quantitative culture was obtained from 43 patients (30 different inpatients and 13 outpatients). The hospitalized patients had received either gentamicin or tobramycin and carbenicillin or ticarcillin parenterally for 3 to 14 days. In addition, some inpatients had been treated with parenteral oxacillin. The outpatients had received three or more of the following antibiotics: tetracycline, oxacillin, sulfasoxazole, cephalexin, and trimethoprim-sulfamethoxazole.

Freshly expectorated sputum was collected in sterile containers and kept at 4°C until cultured. Specimens were subcultured within 1 h of sputum collection. One-half to 1.0 ml of sputum was placed in a sterile glass tube (18 by 150 mm), overlayed with an equal volume of DTT (100 μg/ml), and agitated for 60 s in a Vortex mixer. The final concentration of DTT was 50 μg/ml. After standing at room temperature for 15 min, the sample was cultured qualitatively.

For quantitative cultures, the sample was resuspended, a 1-ml portion was added to 4 ml of phosphate-buffered saline (PBS), and five additional 10-fold dilutions were made. One-tenth milliliter of each of these dilutions was spread onto the following media: 5% sheep blood agar, chocolate agar, MacConkey agar, sodium cetrimide agar, mannitol-salt agar, and Sabouraud dextrose agar. For qualitative cultures, the liquefied sputum was centrifuged at 900 × *g* for 5 min. The supernatant was discarded, and the sediment was cultured by dipping a cotton-tipped swab in the sediment and streaking on the same media as used for quantitative cultures. Sputum which had not been treated with DTT was sampled by dipping a swab into the sputum and streaking on the same media. Plates were incubated at 37°C and were examined at 24 and 48 h. Microorganisms were identified by standard techniques (7).

MICs of gentamicin. Gentamicin MICs for *P. aeruginosa* were determined with a standard microtiter technique (10, 22) by inoculating 10^4 logarithmic-phase organisms into Mueller-Hinton broth supplemented with Ca^{2+} and Mg^{2+} (21).

RESULTS

Antimicrobial activity. The antimicrobial activity of DTT against test strains of *H. influenzae*, *S. aureus*, *P. aeruginosa*, and *C. albicans* is shown in Table 1. *S. aureus* and *C. albicans* were not inhibited by the final concentration of DTT (50 μg/ml); only 4 of 21 strains of *P. aeruginosa* were inhibited by concentrations of DTT near this final strength. However, DTT had inhibitory activity against nontypable *H. influenzae* and *H. influenzae* type b: 16 of 29 strains were inhibited by 31 μg of DTT per ml, and 27 of 29 were inhibited by concentrations of DTT near the final concentration. For all organisms, the MBC was the same as the MIC or one tube dilution higher.

Effect on growth of test strains. At 2 h, 50 μg of DTT per ml slightly inhibited the growth of strains of *P. aeruginosa* and *S. aureus* with MIC and MBC values of 250 μg/ml (representative curves, Fig. 1a and 1b). However, logarithmic growth resumed by 4 h. DTT at 50 μg/ml markedly inhibited growth of *H. influenzae* type

TABLE 1. *In vitro susceptibilities*

Organism (no. tested)	No. of strains inhibited at DTT concn (μg/ml) of:						
	8	16	31.3	62.5	125	250	≥500
P. aeruginosa (rough) (11)	0	0	0	2	3	4	2
P. aeruginosa (mucoid) (10)	0	0	0	2	2	2	4
S. aureus (10)	0	0	0	0	0	2	8
H. influenzae type b (15)	0	0	9	4	2	0	0
H. influenzae non-b (14)	0	0	7	7	0	0	0
C. albicans (4)	0	0	0	0	0	0	4

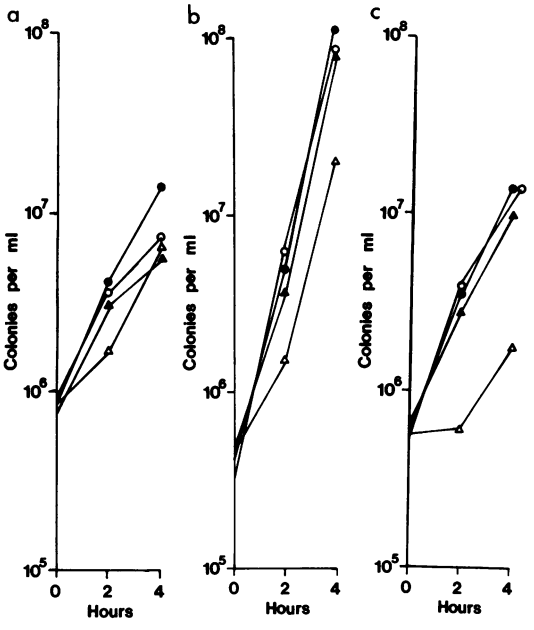

FIG. 1. *Effect of various concentrations of DTT on the growth of representative strains of (a) P. aeruginosa, (b) S. aureus, and (c) H. influenzae type b. Symbols: ●, control; ○, 0.5 μg/ml; ▲, 5 μg/ml; △, 50 μg/ml.*

b (MIC and MBC = 62.5 μg/ml) at 2 h (Fig. 1c), but logarithmic growth was noted at 4 h. Growth of *C. albicans* (MIC and MBC ≥ 500 μg/ml) was not inhibited by the concentrations of DTT tested.

Comparison of simultaneous qualitative cultures of DTT-treated and untreated sputa. Results of cultures of DTT-treated and untreated sputa are shown in Table 2. DTT slightly enhanced recovery of *P. aeruginosa*; it was recovered from 100% of DTT-treated cultures versus 94% of untreated specimens. Neither treated nor untreated sputum cultures were completely successful in detecting *S. aureus*. Only two patients had *H. influenzae* isolated from sputum; *Haemophilus* was recovered both times on routine culture, but from only one of the DTT-treated specimens. *C. albicans* was recovered from 92% of routine cultures, but only from 62% of DTT-treated cultures.

Quantitative sputum cultures. The results of quantitative cultures from 43 patients are summarized in Table 3. Thirty-seven (86%) of the 43 patients were colonized with mucoid strains of *P. aeruginosa* with a median sputum density of 4.3×10^7 CFU/ml; 35 (81%) were colonized with rough strains with a median density of 3.2×10^7 CFU/ml. *S. aureus* was recovered from 15 (35%) of the patients with a median density of 3.5×10^3 CFU/ml. Only one patient had more than 10^7 CFU of *S. aureus* per ml in his sputum; this patient also had the lowest observed density of *P. aeruginosa*, 4.0×10^3 CFU/ml. *H. influenzae* was not recovered from any of the quantitative cultures.

To determine whether appropriate antibiotic therapy has an impact on sputum colonization with *P. aeruginosa*, we compared the density of *Pseudomonas* in the sputum of outpatients with that in hospitalized patients who were receiving parenteral therapy with potent anti-*Pseudomonas* agents. The median sputum density of mucoid *P. aeruginosa* in 26 inpatients who had been treated from 3 to 14 days was 6.4×10^7 CFU/ml; the median density in 11 outpatients was 4.0×10^7 CFU/ml. The median density of rough strains of *P. aeruginosa* was 4.8×10^7 CFU/ml in 26 inpatients and 5.2×10^7 CFU/ml in 10 outpatients. More specific evidence that relatively short-term therapy with anti-*Pseudomonas* agents has a negligible effect on the sputum density of *Pseudomonas* was provided by eight patients whose isolates were available for gentamicin MIC testing. These patients had been receiving gentamicin (as well as carbenicillin or ticarcillin) for a mean duration of 5.3 days (range, 3 to 8 days). The median sputum density of *P. aeruginosa* in these patients was 10^7 CFU/ml (range, 2.4×10^4 to 7.6×10^7 CFU/ml); this is not significantly different from the density observed in outpatients ($P > 0.10$ by Student's t test). All of the *P. aeruginosa* strains isolated from these patients were susceptible to gentamicin in vitro; the mean MIC was 2.7 μg/ml (range, 0.5 to 6.0 μg/ml).

DISCUSSION

Bacteria are not uniformly distributed in

TABLE 2. *Comparison of DTT liquefaction and direct plating for qualitative sputum cultures (36 patients)*

Organism	Total no. of times isolated	Untreated sputum		Sputum liquefied with DTT	
		No. of times isolated	% Recovery	No. of times isolated	% Recovery
P. aeruginosa (rough)	23	21	91	23	100
P. aeruginosa (mucoid)	24	23	96	24	100
S. aureus	11	8	73	9	82
P. mirabilis	4	3	75	4	100
E. coli	4	3	75	3	75
H. influenzae	2	2	100	1	50
C. albicans	13	12	92	8	62

TABLE 3. *Results of quantitative sputum cultures (43 patients)*

Organism	No. of patients with organism	% of patients with organism	Sputum density (CFU/ml)	
			Median	Range
P. aeruginosa (rough)	35	81	3.2×10^7	4.0×10^3–4.0×10^8
P. aeruginosa (mucoid)	37	86	4.3×10^7	1.3×10^6–5.0×10^8
S. aureus	15	35	3.5×10^3	4.0×10^2–2.0×10^7
E. coli	3	7	1.5×10^7	1.2×10^2–4.5×10^7
K. pneumoniae	3	7	1.0×10^3	5.2×10^2–1.2×10^3
P. mirabilis	3	7	1.5×10^7	2.5×10^6–1.0×10^8
P. cepacia	1	2	1.7×10^7	

sputum (13). Therefore, homogenization of sputum has been advocated to reduce sampling error and to increase the accuracy of cultures. To facilitate homogenization and plating of tenacious sputum specimens, such as those characteristically produced by patients with CF, sputum must first be liquefied. Liquefaction and homogenization have not attained widespread acceptance, in part because they are relatively time-consuming procedures, but more importantly, because their superiority to direct plating of CF sputum specimens has not been adequately demonstrated. Kilbourn et al. (6) evaluated sodium 2-ethylhexyl sulfate (Tergemist) in processing sputum from patients with CF and chronic lung disease and found that liquefaction did not enhance recovery of bacteria when compared with routine plating of sputum samples. Other mucolytic agents, such as pancreatin, pancreatin-trypsin, amylase, NAC, and DTT, have not previously been studied in a CF population. NAC, which as been shown to increase recovery of organisms from sputum of patients who do not have CF (14), appears to have in vitro activity against *P. aeruginosa*, an important pathogen in CF. Parry and Neu (16) found that 9 of 13 strains of *P. aeruginosa* were inhibited by 14 μg of NAC per ml, a concentration considerably less than the recommended final concentration of 250 μg/ml.

We were encouraged to study DTT in processing CF sputum specimens because liquefaction of sputum can be performed quickly at room temperature by relatively simple procedures. Moreover, DTT appears to be superior to other mucolytic agents in liquefying sputum (5). However, our studies have not convinced us that liquefaction with DTT has any advantage over direct plating of sputum samples for routine, qualitative clinical work. DTT enhanced the recovery of *P. aeruginosa*, but only slightly. Good recovery of *P. aeruginosa* from DTT-treated specimens was anticipated, since DTT had minimal activity against *Pseudomonas* in our in vitro studies. Although Parry and Neu (16) found that growth of four of five strains of *P. aeruginosa* was inhibited by 0.8% DTT (8 mg/ml), it should be noted that this concentration exceeds the recommended final concentration of 50 μg/ml. Isolation of *C. albicans* was seriously impaired by liquefaction. The poor recovery of *Candida* was probably not due to DTT antimicrobial activity, since representative strains of *Candida* were not inhibited by 500 μg of DTT per ml. We speculated that DTT might enhance aggregation of yeast cells, thus decreasing the sensitivity of subcultures in detecting this organism. However, aggregation was not noted microscopically when *Candida* was grown in BHI broth or in BHI broth containing the recommended final concentration of DTT. Based on these preliminary findings, the poor recovery of *Candida* cannot be explained. Neither DTT-treated nor untreated sputum cultures were completely successful in detecting *S. aureus* and *Enterobacteriaceae*, and liquefaction was not clearly superior to direct plating.

We could not evaluate the effect of DTT on recovery of *H. influenzae* from CF sputum since, in contrast to other investigators (6, 11), we found that very few of our patients (6%) were colonized with this organism. However, the significant in vitro activity of DTT against representative clinical isolates suggests that recovery might be impaired. Twenty-seven of 29 strains of *H. influenzae* were inhibited by 62.5 μg or less of DTT per ml. The inhibitory effect of DTT on the growth of *H. influenzae* was confirmed by our growth curve studies using 50 μg of DTT per ml, although the growth at 4 h of the strains we tested requires further study. Unfortunately, the inhibitory effect of DTT against *H. influenzae* cannot be avoided by using a lower DTT concentration. Since none of our strains had a DTT MIC of <31.3 μg/ml, we assessed the ability of 25 μg of DTT per ml to liquefy sputum from CF patients; we found this final concentration unsatisfactory.

Although liquefaction with DTT does not offer significant advantages in routine clinical work, we found that it greatly facilitates quantitative culturing of tenacious CF sputum. Quan-

titative sputum cultures have been advocated as a means of distinguishing lower respiratory tract infection from pharyngeal colonization (4, 15, 18) and for assessing bacteriological response to antibiotic therapy (9, 15, 18). Generally, bacteria responsible for pulmonary infection are present in a concentration of at least 10^7 organisms per ml of sputum. A recent report suggests that pharyngeal organisms can contaminate coughed sputum in concentrations of $>10^6$ CFU/ml unless sputum specimens are washed before processing (2). However, these investigators did not study CF sputum. CF patients generally are colonized throughout their bronchial tree and have little trouble producing copious amounts of relatively saliva-free sputum, suggesting that washing of sputum samples may not be advantageous in this population. We are currently studying the effect of washing on the recovery of bacteria from clinical specimens submitted to our laboratory.

Quantitative cultures in 43 of our patients revealed that 86% were colonized with mucoid or rough strains of *P. aeruginosa* at median densities of 10^7 CFU/ml. These sputum densities are similar to those reported by Kilbourn et al. (6) and Maguire et al. (11). Thirty-five percent of our patients were colonized with *S. aureus*. The median sputum density of *S. aureus* was 3.5×10^3 CFU/ml, which is strikingly less than the mean sputum density of approximately 10^7 CFU/ml reported by Kilbourn et al. and Maguire et al. Since DTT does not have striking antistaphylococcal activity, this dramatic difference could be a reflection of antibiotic management. The antibiotic management of Kilbourn et al.'s patients was not stated, but the patients studied by Maguire et al. were not treated chronically with antibiotics. All of our patients, whether hospitalized or not, were receiving antibiotics with antistaphylococcal activity at the time of culture.

In contrast, antibiotic therapy did not appear to affect the sputum density of *P. aeruginosa*. The concentrations of both rough and mucoid strains were the same in hospitalized patients, who were receiving gentamicin or tobramycin and carbenicillin or ticarcillin, as in outpatients, who were not receiving antibiotics with significant activity against *Pseudomonas*. Based on antimicrobial susceptibility data in a subgroup of eight hospitalized patients, the failure of antibiotics to reduce the sputum density of *P. aeruginosa* did not appear to be due to bacterial antibiotic resistance. The failure of aminoglycoside therapy to eradicate *P. aeruginosa* from the sputum of patients with CF has been shown previously (12, 17), although quantitative studies were not performed. Crozier and Khan (3) reported eliminating *P. aeruginosa* from the sputum of five CF patients who had received tobramycin, 7.5 mg/kg per day for 7 to 21 days. However, *Pseudomonas* reappeared in the sputum within 1 month after the discontinuation of therapy.

In conclusion, liquefaction and homogenization with DTT do not improve recovery of organisms from routine, qualitative cultures of CF sputum. DTT does appear to be an excellent mucolytic agent for quantitative studies of *S. aureus* and *P. aeruginosa* in CF sputum, although the in vitro activity of DTT against *H. influenzae* may limit its usefulness in studying colonization with this organism. The predominant microorganism colonizing the sputum of CF patients is *P. aeruginosa*, with median sputum densities of approximately 10^7 CFU/ml. The short-term parenteral administration of currently available antibiotics with in vitro activity against *P. aeruginosa* may not have an impact on sputum colonization with this pathogen.

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