SELECTIVE MEDIA FOR THE QUANTITATION OF BACTERIA IN CYSTIC FIBROSIS SPUTUM

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SUMMARY. We used selective media together with aerobic and anaerobic incubation for the quantitation of common pathogens in liquefied sputum from children with cystic fibrosis. The accuracy of the technique was verified by reconstruction studies in which laboratory strains with antibiotic-resistance markers were added to sputum from cystic fibrosis patients. Comparison of the numbers of bacteria found on quantitative culture of clinical specimens with the "predominant" organism found on routine culture yielded a poor correlation. When *Pseudomonas aeruginosa* was the most prevalent on routine culture, it was present in the highest numbers on quantitative culture (mean count = 10^8 cfu/g). However, large numbers of *Haemophilus influenzae* (mean count = 10^7 cfu/g), *Staphylococcus aureus* (mean count = 2×10^6 cfu/g), and streptococci (mean count = 2×10^6 cfu/g) were also present in these cultures. When *S. aureus* was the predominant organism, *H. influenzae* and *P. aeruginosa* were also present in similar numbers (c. 10^7 cfu/g). When *H. influenzae* was the predominant species on routine culture, the mean count was 7×10^6 cfu/g and *P. aeruginosa* was often completely absent. We conclude that the selective technique permits reliable enumeration of sputum bacteria, and offers a more accurate assessment of the microbial flora of sputum in cystic fibrosis than does simple plating of unhomogenised sputum.

INTRODUCTION

Pulmonary infection in children with cystic fibrosis is an important factor in the development of progressive pulmonary insufficiency. It is important to identify the bacteria causing the infection so that appropriate antibiotics can be administered.

The bacteria in infected sputum are not uniformly distributed (May 1953; May, Herrick and Thompson, 1972). Thus, correct identification of the predominant organism depends upon sampling an area representative of infection; moreover, expectorated sputum may be contaminated by oropharyngeal bacteria. Pecora (1959) suggested that transtracheal aspiration of sputum from the lower respiratory tract could circumvent the problem of oropharyngeal contamination. However, this

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procedure is not feasible in children with cystic fibrosis. Washing expectorated sputum with saline prior to culture and identification may help to separate true pathogens from upper respiratory tract commensals (Lapinski, Flakas and Taylor, 1964; Bartlett and Finegold, 1978). Alternatively, quantitative sputum cultures may be useful (Louria, 1962). This technique assumes that the infecting bacteria from patients with pneumonia are present in sputum in concentrations that are several hundred-fold greater than in saliva (Monroe *et al.*, 1969).

Kilbourn *et al.* (1968) introduced a quantitative culture technique for cystic fibrosis sputum. After liquefaction of the sputum, dilutions were plated on media permitting selective growth of gram-negative bacilli and staphylococci. This technique permitted accurate determination of the numbers of certain bacteria (Hammerschlag *et al.*, 1980). However, fastidious organisms such as *Streptococcus pneumoniae* or *Haemophilus influenzae* were often difficult to identify in the presence of aerobic gram-negative bacilli. Selective media for these organisms have been developed (Huang, Van Loon and Sheng, 1961; Sondag *et al.*, 1977; Roberts and Cole, 1980) which should improve the isolation rate of these and other potential pathogens.

In this report we describe a quantitative sputum culture procedure using five selective media together with aerobic and anaerobic incubation for the isolation and quantitation of sputum bacteria.

MATERIALS AND METHODS

Bacterial strains. The reference bacterial strains used were: *Pseudomonas aeruginosa* PAO 236 (nalidixic acid resistant and non-mucoid) by courtesy of Dr S. Falkow, Stanford University; *Haemophilus influenzae* E₁₅ (streptomycin and erythromycin resistant) developed in this laboratory; *Staphylococcus aureus* R549 (methicillin resistant) by courtesy of Dr A. Evans, University of Washington; and *Streptococcus pneumoniae* R550 (penicillin resistant) by courtesy of Dr L. Tompkins, University of Washington. *P. cepacia* ATCC strains 10856, 25609, 25608, *P. maltophilia* ATCC13637, *P. putida* ATCC12644, and *P. alcaligenes* ATCC14909 were purchased from the American Type Culture Collection, Rockville, MD. Dr L. Tompkins also supplied two clinical isolates of *P. cepacia*. Twenty-eight noncapsulate *H. influenzae* strains isolated from the sputum of adults were supplied by Dr P. Piot, Antwerp, Belgium. An additional 12 *H. influenzae* type b strains, isolated from the cerebrospinal fluid of children, were from our own laboratory. All test strains were identified according to standard criteria (Lennette *et al.*, 1980); *H. influenzae* strains were shown to require haemin and β -nicotinamide adeninedinucleotide for growth on Brain-Heart Infusion Agar (Difco).

Antibiotics. Neomycin and bacitracin (Upjohn, Kalamazoo, MI.), gentamicin (Schering Corp., Bloomfield, NJ.), nalidixic acid (Calbiochem, La Jolla, CA.), methicillin, penicillin G and erythromycin base (Sigma Chemical Corp., St Louis, MO.) and streptomycin (Parke Davis, Detroit, MI.) were obtained from the manufacturers.

Reagents. L-histidine and equine haemin hydrochloride were obtained from Calbiochem Corp., β -nicotinamide adeninedinucleotide (NAD⁺) and ethylhydrocupreine (optochin) were purchased from Sigma Chemical Corp. and sodium dodecylsulphate (SDS) was purchased from Matheson Coleman and Bell, Norwood, Ohio.

Media. Cetrimide agar (Difco) for identification of *Pseudomonas* and MacConkey agar (Difco) for identification of other gram-negative bacilli were prepared according to the manufacturer's instructions. Blood-agar plates were prepared by adding 5% sterile sheep blood to autoclaved Trypticase Soy Agar (TSA; BBL). Certain plates were supplemented with gentamicin 10 μ g/ml and neomycin 25 μ g/ml (BA n/g) to select for streptococci. Modified Brain Heart Infusion Agar (mBHI) was prepared according to the manufacturer's instructions and supplemented with L-histidine 10 μ g/ml, haemin 10 μ g/ml, NAD⁺ 10 μ g/ml and bacitracin 250 μ g/ml for the selective isolation of *Haemophilus* spp.

Mannitol-salt agar plates and Dextrose-soy agar plates containing cycloheximide 400 µg/ml and chloramphenicol 50 µg/ml (DSA c/c) for isolation of yeasts, were purchased from Prepared Media Laboratory, Renton, Washington.

For recovery of antibiotic-resistant reference strains, cetrimide agar was supplemented with nalidixic acid 200 µg/ml (*Pseudomonas*), BA n/g was supplemented with penicillin G 0.5 µg/ml (*Streptococcus*), TSA was supplemented with methicillin 20 µg/ml (*Staphylococcus*) and mBHI was supplemented with streptomycin 250 µg and erythromycin 10 µg/ml (*Haemophilus*).

Reconstruction experiments. Sputum collected from each of three cystic fibrosis patients during a 12-h period was pooled and the numbers of bacteria were determined by the technique described below. The samples of sputum from these patients were selected on the basis of previous qualitative cultures. One contained 10^6 mucoid *P. aeruginosa*/g, one contained 10^6 *S. aureus*/g and the third pool contained <200 cfu of *P. aeruginosa* or *S. aureus*/g. Antibiotic resistant *P. aeruginosa*, *Str. pneumoniae*, *H. influenzae* or *S. aureus* were added to portions of each liquefied pool to achieve a concentration of 10^6 , 10^4 or 10^2 cfu/ml and the samples were mixed by stirring; these samples were then processed by the quantitative technique on antibiotic-supplemented selective media.

Quantitation of sputum bacteria. Dithiothreitol (Sputalysin; Calbiochem) was diluted 1 in 10 with sterile phosphate-buffered saline (128.3 mM NaCl, 2.8 mM KCl, 0.6 mM sodium-potassium phosphate, pH 7.4 ± 0.05) containing sterile gelatin 0.1% (PBS-G) yielding a final concentration of dithiothreitol 50 µg/ml; 0.6 ml of the diluted dithiothreitol was mixed with an equal volume of each sputum sample and the mixture was stirred for 5 min at room temperature to liquefy the sputum. The homogenate was further diluted 1 in 10, 1 in 100, and 1 in 10 000 with PBS-G and 0.1 ml of each dilution was spread with a sterile glass rod on the surface of each of the five media (figure). The appropriate antibiotic-supplemented medium was used for identification and quantitation of the antibiotic-resistant test organisms that had been added to the sputum pools. BA (n/g) and mBHI were incubated anaerobically in a GasPak (BBL) jar to prevent growth of *Pseudomonas* spp. and to enhance haemolysis by streptococci. All plates were incubated at 37°C for 24–48 h. After incubation, organisms were provisionally identified by colonial morphology and colour (Lenette *et al.*, 1980); presumptive confirmation of identity was accomplished by subculture to additional selective media. Two different techniques of subculturing were employed—replica plating and streaking with disposable sterile toothpicks.

Replica plating was by the velvet pad technique (Lederberg and Lederberg, 1952). After aerobic incubation of the replica plate at 37°C for 18–24 h, colonial morphology and colour were again used for identification. When there were >200 colonies on the primary plate, representative colonies were sampled with toothpicks and streaked in a radial fashion on the secondary selective media.

Gram-stained films were made of organisms growing on the MacConkey and blood agar plates replicated from the mBHI to verify that they were gram negative and of non-haemolytic colonies growing on the BA (n/g) plate to confirm that they were streptococci.

Source of sputum samples. Children with cystic fibrosis seen in the outpatient clinic, or those admitted to hospital for parenteral antibiotic therapy, expectorated into sterile, weighed sputum cups. The samples were immediately transported to the clinical bacteriology laboratory for routine culture after which they were liquefied and cultured by the quantitative technique. To determine the effect of storage, 20 samples whose weight exceeded 2.5 g were stirred vigorously until they appeared homogeneous, and a 0.5 g portion was cultured quantitatively. The remainder was stored at 4°C; 24 and 48 h later, the refrigerated specimen was again cultured to determine the effect of storage on the density of viable organisms. Ninety-nine consecutive sputum samples were collected; 60 were also processed in the routine clinical bacteriology laboratory to compare the quantitative technique to routine culture.

RESULTS

This investigation concentrated on the isolation of *P. aeruginosa*, *S. aureus*, *Str. pneumoniae* and *H. influenzae* because they are the chief pulmonary pathogens in

Step 1: Homogenise sputum with dithiothreitol (Sputalysin)

Step 2: Dilute 1 in 10, 1 in 100 and 1 in 10 000

Step 3: Plate 0.1 ml of undiluted homogenate and of each dilution on to:*

(i) Cetrimide agar	— growth = <i>Pseudomonas</i> sp.
(ii) Blood agar (n/g)	— α -haemolysis = <i>Str. pneumoniae</i> or viridans streptococci
(incubate anaerobically)	— β -haemolysis = β -haemolytic streptococci or staphylococci
	— no haemolysis = non-haemolytic streptococci, staphylococci, gram-negative bacilli or yeast
(iii) mannitol salt agar	— yellow = <i>S. aureus</i> or yeast
	— pink = <i>S. epidermidis</i>
(iv) Modified BHI agar	— growth = <i>H. influenzae</i> etc.
(incubate anaerobically)	
(v) Dextrose soy agar (c/c)	— growth = <i>Candida</i> sp.

Step 4: Confirm identity of isolates with gram-film and supplementary tests as necessary

* A MacConkey agar plate should be included for specimens from patients suspected of harbouring Enterobacteriaceae.

FIG.—Steps for processing cystic fibrosis sputum for quantitative culture of common bacteria after overnight incubation at 37°C. Preliminary identification is made on the basis of colonial morphology.

patients with cystic fibrosis (Huang *et al.*, 1961; Iacocca, Sibinga and Barbero, 1963). Cetrimide plates were relatively specific for the growth of *P. aeruginosa*. *P. putida* produced small (1.0 mm diam.), faintly yellow, opaque colonies that had a thick “buttery” consistency. All five strains of *P. cepacia* grew on cetrimide plates but produced small (< 1.0 mm diam.) opaque white colonies with an ammoniacal odour. *P. alcaligenes* and *P. maltophilia* did not grow on cetrimide agar. There was delayed growth of *P. aeruginosa* from sputum cultures obtained from patients receiving antibiotics active against that species; in 27 of 37 consecutive isolates, growth on cetrimide agar was evident only after incubation for 48 h. None of the *Pseudomonas* spp. grew anaerobically on the supplemented BHI agar.

Sixty-three *S. aureus* isolates identified by a positive coagulase test produced yellow colonies, 1–2 mm diam., on mannitol salt agar after incubation for 48 h at 37°C. *S. epidermidis* (coagulase negative) produced small, dry, pink colonies on mannitol salt agar. Of 60 consecutive sputum cultures plated on this media, low numbers (< 10² cfu/g) of a small gram-positive coccus that appeared as large, pink mucoid colonies grew from two. These organisms were subsequently identified as micrococci.

Blood agar supplemented with neomycin and gentamicin inhibited the growth of most oropharyngeal gram-positive and gram-negative genera other than streptococci. Anaerobic incubation made it easier to identify the species by enhancing haemolysis (Sondag *et al.*, 1977).

For α -haemolytic streptococci, growth on ethylhydrocupreine 5 μ g/ml or sodium dodecyl sulphate 1% was used to differentiate viridans streptococci from *Str. pneumoniae*. Typing sera were used to group the β -haemolytic streptococci. Further identification of α -haemolytic streptococci was not undertaken.

Brain heart infusion agar supplemented with bacitracin and incubated anaerobically was highly selective for *H. influenzae*. All other organisms that grew on this medium could be distinguished from *H. influenzae* by colony size ($\geq$ 2 mm diam.) and consistency (smooth to mucoid); all such bacteria grew aerobically on MacConkey agar and were *Escherichia coli*, *Enterobacter* spp. or *Klebsiella pneumoniae*.

TABLE I

Recovery of bacteria by quantitative culture of reconstructed sputum

Test species	Percentage recovery when the inoculum contained		
	10 ⁶ cfu/ml	10 ⁴ cfu/ml	10 ² cfu/ml
<i>P. aeruginosa</i>	100	80	82
<i>Str. pneumoniae</i>	100	100	82
<i>H. influenzae</i>	95	94	94
<i>S. aureus</i>	100	100	97

Reconstruction experiments

The three sputum pools gave similar recovery rates for all four organisms. Therefore, all three results were combined (table I). For sputum seeded with 10⁶ organisms/g, there was 95–100% recovery of each organism. At a density of 10² organisms/g, the recovery rate was lower, but still greater than 80%. Similar recovery rates were found with each of the organisms tested. All 40 *H. influenzae* strains tested grew anaerobically on the BHI-bacitracin agar; incubation for 36–48 h was necessary for the typical morphology to be apparent.

There was no detectable change in the numbers of *S. aureus*, *P. aeruginosa*, *H. influenzae* and *Str. pneumoniae* isolated after storage for 2 days at 4°C; longer storage conditions were not tested.

Clinical specimens

The results of quantitative culture and direct plating of unhomogenised sputum were strikingly different (table II). For the 60 sputum samples examined, there was little relationship between the predominant organism isolated on routine culture, and the number of each species isolated on selective media by the quantitative method. However, certain trends were evident. When *H. influenzae* was detected as the primary isolate on routine culture, small numbers of *P. aeruginosa* were isolated by the quantitative technique, although *H. influenzae* was more numerous. When *P. aeruginosa* was the predominant organism on routine culture, it was also most numerous on quantitative culture; however, the presence of *P. aeruginosa* did not

TABLE II

Comparison of routine and quantitative culture of sputum from children with cystic fibrosis

"Primary organism" on routine culture	Number of specimens	Mean number of cfu (range)/g of sputum of			
		<i>P. aeruginosa</i>	<i>H. influenzae</i>	<i>S. aureus</i>	streptococcus
<i>P. aeruginosa</i>	22	1 × 10 ⁸ (4 × 10 ⁴ –8 × 10 ⁸)	1 × 10 ⁷ (3 × 10 ⁶ –7 × 10 ⁷)	2 × 10 ⁶ (5 × 10 ⁵ –1 × 10 ⁷)	1 × 10 ⁶ (4 × 10 ² –6 × 10 ⁶)
<i>H. influenzae</i>	4	5 (0–22)	7 × 10 ⁶ (4 × 10 ³ –3 × 10 ⁷)	1 × 10 ⁵ (1 × 10 ⁴ –5 × 10 ⁵)	7 × 10 ⁵ (8 × 10 ⁴ –2 × 10 ⁶)
<i>S. aureus</i>	18	4 × 10 ⁷ (2 × 10 ³ –1 × 10 ⁸)	1 × 10 ⁷ (2 × 10 ⁵ –1 × 10 ⁸)	1 × 10 ⁷ (2 × 10 ⁵ –4 × 10 ⁷)	2 × 10 ⁵ (2 × 10 ⁴ –8 × 10 ⁶)
streptococci	16	1 × 10 ⁵ (2 × 10 ² –6 × 10 ⁸)	5 × 10 ⁵ (5 × 10 ⁴ –3 × 10 ⁶)	2 × 10 ⁵ (8 × 10 ³ –1 × 10 ⁶)	2 × 10 ⁶ (2 × 10 ⁴ –8 × 10 ⁶)

exclude large numbers of *H. influenzae* (10^7 cfu/g), *S. aureus* (10^6 cfu/g) and streptococci (10^6 cfu/g).

Streptococci were isolated from every sputum sample, and were present in slightly higher numbers (10^6 versus 10^5) when they were identified as the "primary organism" on routine culture. Only 12 out of 73 streptococci isolated were *Str. pneumoniae* and numbers of this species did not exceed 10^4 cfu/g. In contrast, quantitative culture of the 60 consecutive sputum samples yielded *S. aureus* (15 samples), *P. aeruginosa* (16 samples), and *H. influenzae* (6 samples) in concentrations $\geq 10^5$ cfu/g. Sixteen samples contained $\geq 10^5$ cfu/g of *S. aureus* and *P. aeruginosa*.

DISCUSSION

The technique described by Kilbourn *et al.* (1968) took account of the non-uniform distribution of bacteria in sputum and used two selective media for identification of *Staphylococcus* spp. and gram-negative bacilli. More recently, George, Gillett and Roycroft (1980) and Roberts and Cole (1980) noted that, in the presence of *Pseudomonas* spp., other species, particularly of the genera *Haemophilus* or *Streptococcus*, were rarely isolated. As a result, selective techniques were devised for the isolation of streptococci or *Haemophilus* spp. in the presence of *Pseudomonas* spp.

We have combined dithiothreitol liquefaction and dilution to quantitate sputum bacteria and have used five selective media together with aerobic and anaerobic incubation to facilitate isolation. The media were chosen to identify the organisms most commonly isolated from sputum in cystic fibrosis: *S. aureus*, *Pseudomonas* spp., *H. influenzae*, streptococci, *Proteus* spp., yeasts or coliform bacilli (Feigelson and Pecau, 1967; Huang *et al.*, 1961; Iacocca *et al.*, 1963). Although the aminoglycoside-supplemented blood agar plate had the potential to grow the greatest number of bacteria, we found the number of genera other than *Streptococcus* limited.

We feel that this method reliably quantitates the organisms most commonly found in sputum from children with cystic fibrosis, and offers additional information in comparison to the standard plating method. Future studies will determine the value of quantitative sputum cultures in children with cystic fibrosis.

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