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Utility of Gram Stain in Evaluation of Sputa from Patients with Cystic Fibrosis

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The utility of sputum Gram stain in assessing salivary contamination and in predicting the presence of pathogens on the basis of morphology was investigated in 287 respiratory specimens from patients with cystic fibrosis. Where acceptability for culture was defined as a leukocyte/squamous epithelial cell ratio of >5, 76.6% (220 of 287) of respiratory specimens received in the laboratory were considered acceptable. Unacceptable specimens were more common in younger patients. The positive predictive value of the Gram stain for growth from acceptable sputum samples was 98% for *Pseudomonas aeruginosa*, 84.4% for *Pseudomonas cepacia*, 86.3% for *Staphylococcus aureus*, and 100% for *Haemophilus influenzae*. In cystic fibrosis patients, as has been reported for respiratory specimens in general, Gram stain of respiratory specimens is helpful for interpreting culture results.

Gram stain of sputum is established as an important component of the bacteriological investigation of lower respiratory tract infection (2, 14). With microscopy at lower power (×100), the degree of salivary contamination and thus the suitability of the specimen for culture can be assessed, and at higher power under oil immersion (×1,000), presumptive identification of pathogenic bacteria can be made. From the laboratory standpoint, examination of a stained sputum smear can be of further value in enhancing the quality control of culture media used for the primary isolation of specific bacteria.

Cystic fibrosis (CF) is the most common lethal genetic disorder in Caucasians (6). Progressive pulmonary disease commonly associated with pulmonary infection is the principal cause of morbidity and mortality in CF (18). Yet, there is little published information on the value of the Gram stain in the bacteriological workup of sputum submitted from patients with this disorder. Its possible role in assessing sample quality or in presumptively identifying the major CF respiratory pathogens (*Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and, in many medical centers, *Pseudomonas cepacia*) has yet to be defined.

The purpose of the present study was therefore to evaluate the utility of the Gram stain in assessing the degree of salivary contamination of CF respiratory specimens and in predicting the presence of specific pathogens in culture on the basis of staining and cellular morphological characteristics.

MATERIALS AND METHODS

Patients. The Hospital for Sick Children, Toronto, Canada, has a daily outpatient clinic that serviced 579 patients with CF at the time the study was carried out. To facilitate specimen acquisition and processing, specimens collected on two clinic days (Tuesday and Wednesday) were designated for the study. All patients seen on these two clinic days during November and December 1991 and March and June 1992 from whom a lower respiratory tract specimen was obtained were included in the study.

Specimen collection and processing. Expectorated sputum samples were collected in sterile plastic containers from patients 6 years of age and older and transported immediately to the laboratory for processing. Two hundred eighty-seven specimens were available for study from 270 patients. A nasopharyngeal aspirate was submitted from patients less than 6 years of age. This was performed by inserting an 8 French 15-in. (ca. 38-cm) feeding tube (Baxter Healthcare Corporation, Deerfield, Ill.) pernasally until resistance was felt, or it was estimated that the posterior nasopharynx had been reached, and aspirating back with a 60-ml syringe. The feeding tube was then immediately sent to the laboratory in a sterile container; on receipt, the aspirate was expelled with air from the feeding tube into the sterile container for further examination.

In the laboratory, specimens were examined macroscopically for the presence of pus or mucus, a Gram stain was performed from the most purulent portion of the specimen, and a purulent portion of the specimen was then cultured onto the following primary isolation media: (i) Columbia blood agar base (Quelab Laboratories, Inc., Montreal, Quebec) with 5% horse blood; (ii) bile salts agar (Oxoid MacConkey agar; Unipath Ltd., Basingstoke, Hampshire, England); (iii) chocolate agar containing GC agar base (Difco Laboratories, Detroit, Mich.) and Oxoid agar (Unipath Ltd.; in combination), 10% horse blood, and 1% CoFactor Enrichment (Quiger Laboratory, West Sacramento, Calif.); and (iv) bile salts agar (described above) with 5 mg of polymyxin B sulfate (Burroughs Wellcome, Inc., Kirkland, Quebec) per liter.

All plates were incubated for 48 h at 37°C in 5% CO_2. On examination of the plates, potential pathogens, including *S. aureus*, *H. influenzae*, members of the family *Enterobacteriaceae*, *P. aeruginosa*, *P. cepacia*, and other glucose nonfermenters (including *Xanthomonas maltophilia*) as well as fungi were isolated and identified by routine techniques (14).

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TABLE 1. Age distribution of CF patients in study

Age group (yr)	CF patients	
	No.	%
Preschool children (0–5)	25	8.7
Primary school children (6–12)	56	19.5
Teenagers (13–19)	58	20.2
Young adults (20–29)	75	26.1
Adults (>29)	73	25.4

For the purpose of this study, data on *S. aureus*, *H. influenzae*, *P. aeruginosa*, and *P. cepacia* were analyzed.

Microscopic examination. The Gram-stained smears were examined microscopically by one of us (E.S.) for the presence of leukocytes (LC), squamous epithelial cells (SEC), and specific bacterial morphotypes (see below). The LC and SEC counts were estimated after the review of several fields at both ×100 and ×1,000 magnifications, and the average LC/SEC ratio was recorded. A ratio of >5 was considered to represent a suitable sample by the criteria of Kalin et al. (17).

Further examination of the smear under oil immersion was performed to identify four specific bacterial morphotypes: (i) slender straight gram-negative rods, (ii) short ovoid gram-negative rods with bipolar staining, (iii) gram-positive cocci in clusters, and (iv) small pleomorphic gram-negative bacilli. Each of the four cell morphotypes was correlated with the presence in culture of the recognized CF pathogens *P. aeruginosa*, *P. cepacia*, *S. aureus*, and *H. influenzae*, respectively.

RESULTS

Two hundred eight-seven specimens from 270 patients were processed as part of the study. The study population consisted of 139 males and 131 females (male/female ratio, 1.1:1), ranging in age from 3 months to 52 years of age, in the age categories shown in Table 1.

On macroscopic examination, 221 (77%) of the respiratory specimens submitted were purulent, and the remaining 66 (23%) specimens were nonpurulent. There was good correlation between the LC/SEC ratio and gross purulence; Fig. 1 displays the correlation of macroscopic examination and LC/SEC ratio with age.

Further work to define the prevalence of known bacterial pathogens and to assess the role of the Gram stain in their identification in stained smears was done on the 220 specimens with an LC/SEC ratio of >5 at ×1,000 magnification. *P. aeruginosa* was the most common respiratory pathogen isolated (130 of 220, 60%), and 45% (99 of 220) of specimens grew *P. cepacia*. *S. aureus* and *H. influenzae* were isolated from 15.9% (35 of 220) and 13.6% (30 of 220) of specimens, respectively. At least two of these pathogens were isolated from approximately 42% of specimens; in the remaining specimens, only one of the isolates under study was cultured. The age-related frequency of distribution of the organisms is shown in Fig. 2.

In 12 of the 220 specimens (0.6%), coliforms were isolated (*Enterobacter* spp., 4; *Proteus* spp., 3; *Escherichia coli*, 3; *Serratia* spp., 2), and in 6 specimens (0.3%), *X. maltophilia* was isolated. *Aspergillus* spp. were isolated in 16 specimens (7.4%), and *Candida* spp. were isolated in 2 specimens (0.9%).

As can be seen in Tables 2 to 5, in the 220 acceptable specimens, the examination of Gram-stained smears was

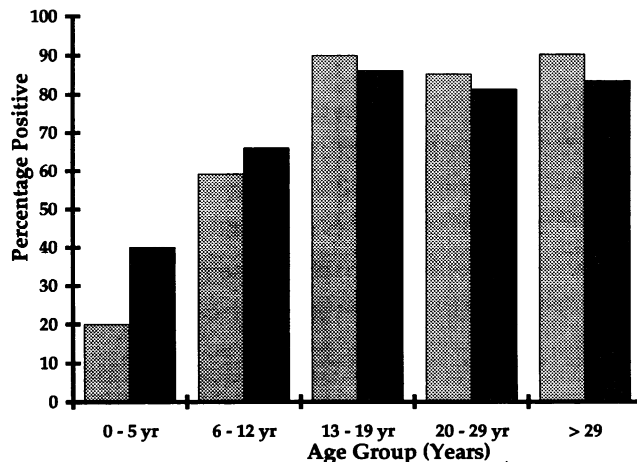

FIG. 1. Gross and microscopic examination of respiratory specimens from CF patients by age groups. Gross purulence correlates with the acceptability of a specimen by using an LC/SEC ratio of >5, and these characteristics occur more frequently in the older patient population. Symbols: ▨, purulent; ■, LC/SEC = >5.

most sensitive in predicting laboratory isolation of *P. aeruginosa* (sensitivity, 87.2%). The specificity of the Gram stain appearance of all four morphotypes in this group of specimens was high, reaching 100% for *H. influenzae*, with a low of 87.7% for *P. cepacia*. The positive and negative predictive values of the Gram stain for isolation of the organism in culture was highest for *H. influenzae* but was above 80% for all pathogens studied.

DISCUSSION

The value of the sputum Gram stain in patients with suspected lower respiratory tract infection has traditionally been in effecting a cost-effective screen for oropharyngeal contamination of the specimen (12, 17, 23) and in determining the etiology of the infection (8, 26) to guide empiric antibiotic therapy. The quality and utility of the Gram stain assessment have been shown to depend on both collection patterns for procuring the specimen (16) and on the expertise of the personnel in preparing and interpreting the Gram stain (5). Despite some limitations, the potential benefits to be gained by sputum Gram staining have made this test a cornerstone of the bacteriological assessment of sputum samples (14).

Although evaluation of the Gram stain to determine specimen acceptability is based on an assessment of LC and SEC, the optimal formula to guide specimen suitability has not been determined. Bartlett et al., Murray and Washington, and Van Scoy have suggested rejection of the specimen on the basis of the absolute number of SEC and/or LC per microscopic field (2, 23, 28), whereas Heinemann and Radano and Kalin et al. based specimen acceptability on the LC/SEC ratio (12, 17). The advantage of the latter approach is that the assessment by ratio compensates for differences in the thickness of the smear or in the uneven distribution of cells within the preparation.

In CF, a genetic defect results in a defective transmembrane regulator that leads to abnormal chloride transport across exocrine glands and secretory epithelia (22). Pulmonary secretions are dehydrated and thick, becoming hyperviscous during superinfections (7, 24). High concentrations

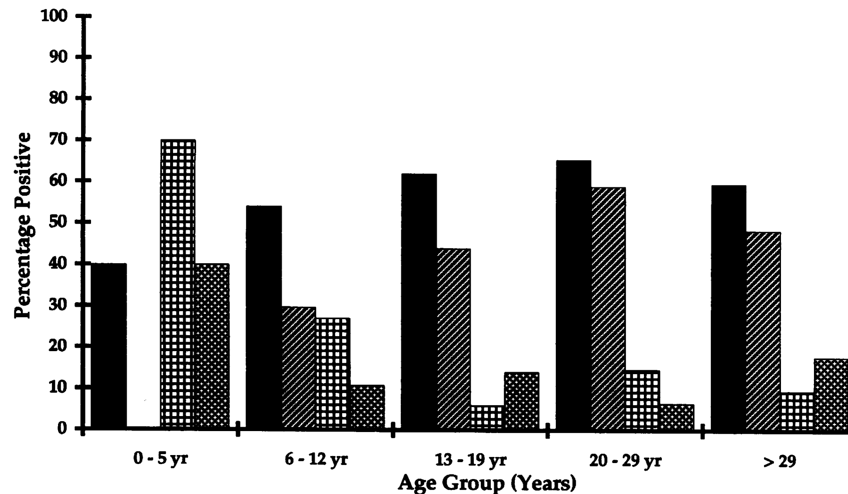

FIG. 2. Age-related distribution of *P. aeruginosa* (▩), *P. cepacia* (■), *S. aureus* (▦), *S. aureus* (▩) and *H. influenzae* are more prevalent in younger patients, whereas *P. aeruginosa* and *P. cepacia* are more prevalent in older children and adults.

of DNA are present in the pulmonary secretions and are primarily LC derived (13), correlating with chronic infection. Sputum production in older patients with CF is copious, and Gilligan has stated that "obtaining sputum that meets the criteria for a good specimen, i.e., a >1 ratio of white blood cells/epithelial cells on Gram stain, is easily accomplished" (9). In a study comparing conformity of bacterial growth in sputum and contamination-free endobronchial samples, Gilljam et al. anecdotally reported that all CF patients for whom a Gram-stained smear was examined had LC/SEC ratios of >5 (10). However, there has been no systematic evaluation of the Gram stain in lower respiratory specimens submitted from CF patients and in particular from young CF patients.

In our study, by using the criteria of Kalin et al. 76.6% of specimens were acceptable for culture, a result similar to that initially reported by the other authors. There was a direct relationship between suitability for culture and age, increasing from 40% in children up to 5 years of age to 66 to 86% in older children and adults. In the older patients, this can likely be explained by the nature of the patients seen in our CF clinic, which includes patients with pulmonary exacerbation as well as patients with stable or mild pulmonary disease. In the younger patients, the use of the nasopharyngeal aspirate to assess respiratory flora may be responsible for suboptimal specimen procurement. Different methods for sampling the lower respiratory tract in children with CF, including throat swab (19, 25), "gagged sputum" (9, 29), and nasopharyngeal aspirate have been reported. The predictive value of positive throat swab and gagged-sputum cultures compared with that of culture of bronchial secretions and bronchoalveolar lavage specimens has been limited, and it is likely that nasopharyngeal aspirate culture is similarly limited. Despite these limitations, however, it is difficult to justify the routine use of more invasive methods of sampling lower respiratory flora, and these indirect methods will for the most part remain the norm.

Our study also examined the ability of the sputum Gram stain to predict the growth of respiratory pathogens in CF specimens. In reviewing prior studies examining the accuracy of the Gram stain in identifying pneumococci in sputum from patients with pneumonia, Rein et al. found a broad range of results, including a sensitivity of 50 to 96% and specificity of 12 to 100% (26). Fine et al. recently reported that the house staff's Gram stain interpretations displayed sensitivities of 86 and 80%, specificities of 72 and 88%, and positive predictive values of 43 and 73% for *Streptococcus pneumoniae* and *H. influenzae*, respectively, compared with those of further microbiological evaluation (5). These data suggested that there may be some utility to predicting bacterial growth in CF specimens on the basis of sputum Gram stain.

We focused on the Gram stain appearance and growth of *S. aureus*, *H. influenzae*, *P. aeruginosa*, and *P. cepacia* because of the prevalence of these particular organisms in CF sputum (9, 21). The probability that a CF patient with a sputum Gram stain identifying specific pathogens indeed has those pathogens in the sputum depends on both the accuracy of the tests and the prevalence of the organisms in the sputa studied. In our patient population, *P. aeruginosa*, followed by *P. cepacia*, was the most common pathogen isolated from

TABLE 2. Relationship between Gram stain findings and culture results of *P. aeruginosa* in sputum specimens from CF patients[a]

Slender gram-negative rods	No. of cultures		Total no. of cultures
	Positive	Negative	
Present	116	2	118
Absent	17	85	102
Total	133	87	220

[a] Sensitivity, 87.2%; specificity, 97.7%; positive predictive value, 98%; negative predictive value, 83.3%.

TABLE 3. Relationship between Gram stain findings and culture results of *P. cepacia* in sputum specimens from CF patients[a]

Gram-negative oval rods with bipolar staining	No. of cultures		Total no. of cultures
	Positive	Negative	
Present	81	15	96
Absent	17	107	124
Total	98	122	220

[a] Sensitivity, 82.6%; specificity, 87.7%; positive predictive value, 84.4%; negative predictive value, 86.3%.

TABLE 4. Relationship between Gram stain findings and culture results of *S. aureus* in sputum specimens from CF patients[a]

Gram-positive cocci in clusters	No. of cultures		Total no. of cultures
	Positive	Negative	
Present	19	3	22
Absent	16	182	198
Total	35	185	220

[a] Sensitivity, 56.6%; specificity, 98.3%; positive predictive value, 86.3%; negative predictive value, 92%.

acceptable sputa. Similar to previous reports (1, 4), *S. aureus* and *H. influenzae* were more frequently isolated from infants and younger children. Colonization with *P. cepacia* was absent in patients 5 years of age and younger but increased to about 60% for young adults. The overall *P. cepacia* colonization rate of 45% is a marked increase from an early report from our center which described colonization rates of 9% in children less than 10 years of age and 21% in patients older than 10 years (4, 15).

Preliminary observations in our laboratory revealed that Gram stains of several CF sputum samples contained numerous short oval gram-negative rods with bipolar staining, with the so-called safety-pin appearance, similar to the cellular morphology of pure cultures of many *P. cepacia* isolates from our CF patients. This morphology is not specific for *P. cepacia*, having been described for other organisms such as *Pasteurella* species. Of interest, however, is that bipolar staining with methylene blue or Wright's stain has been described for *Pseudomonas pseudomallei*, a pseudomonad that, like *P. cepacia*, is a member of rRNA homology group II (27). Whether *Pseudomonas gladioli*, another rRNA group II pseudomonad that has been identified in CF sputum (3), displays this morphology has not been studied. Our data, in combination with those cited above, suggest that the bipolar morphology may be helpful in predicting the presence of certain pseudomonads.

In our study, the presence of numerous slender gram-negative rods in the Gram stain correlated with the presence of *P. aeruginosa* in culture. Although this morphology is not unique, in our group of CF patients, such an appearance was highly predictive. We would like to emphasize, however, that the strong correlation in our study between slender gram-negative rods and *P. aeruginosa* and between safety-pin morphology and *P. cepacia* may be in part related to the low prevalence of coliforms and nonfermentative organisms such as *X. maltophilia* in the sputa of our CF patient population. We would advise other laboratories that service CF patients to assess the prevalence of the various respiratory pathogens in their own population prior to extrapolating directly from our results.

TABLE 5. Relationship between Gram stain findings and culture results of *H. influenzae* in sputum specimens from CF patients[a]

Pleomorphic gram-negative coccobacilli	No. of cultures		Total no. of cultures
	Positive	Negative	
Present	23	0	23
Absent	7	190	197
Total	30	190	220

[a] Sensitivity, 76%; specificity, 100%; positive predictive value, 100%; negative predictive value, 96.4%.

We found that the sputum Gram stain displayed reasonable and often excellent sensitivity, specificity, and predictive values compared with culture for identifying *P. aeruginosa*, *P. cepacia*, *S. aureus*, and *H. influenzae* in acceptable CF sputa. The inherent lack of sensitivity of microscopic examination when compared with culture is likely responsible for the limited sensitivities in identifying the target organisms in our study, ranging from 56.5 to 87.2%. The notably low sensitivity for *S. aureus* (56.5%) may further be explained by lower numbers of *S. aureus* than of *P. aeruginosa* in the sputa of our patients as has been described in another series of CF sputum samples (11). Approximately 80% of our patients were on antibiotic therapy, potentially rendering visible organisms nonviable, and this may have influenced the specificity and positive predictive value of the Gram stain compared with culture. The atypical morphology of organisms exposed to antibiotics may have had an additional impact the results (20).

We have not attempted in this study to correlate Gram stain morphology with clinical presentation (e.g., acute pulmonary exacerbation versus stable pulmonary disease) nor is our goal to extrapolate our findings to respiratory specimens from non-CF populations. We have demonstrated that in sputum from CF patients, the Gram stain appearance of selected pathogens is highly predictive of their presence. The safety-pin morphology of *P. cepacia* was a unique finding; in this group of patients, the positive predictive value of this stained morphology was 84.4%. Further work demonstrated that objective criteria developed to assess specimen acceptability could be useful in separating out specimens procured from infants and young children by nasopharyngeal aspirate and yet highlighted the fact that even older CF patients may submit suboptimal respiratory specimens.

The sputum Gram stain is a rapid, inexpensive, and easily accessible microbiological tool. Questions regarding the place of the Gram stain in the bacteriological workup of CF sputa include the following. (i) Should the Gram stain be routinely performed in these specimens? (ii) What is the correlation between Gram stain appearance and clinical status? This study was not intended to answer these two questions. On the other hand, the fundamental clinical microbiological principle of examining clinical specimens by microscopy as well as by culture may justify the use of sputum Gram stain in routine practice.

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