

Evaluation of some methods for the laboratory examination of sputum

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SUMMARY Methods for the quantitation of leucocytes, squamous epithelial cells, and potential pathogens in sputa are described. Microscopic examination showed that 58% of sputa tested (554/957) were purulent or moderately purulent and 48% were moderately or heavily contaminated by squamous epithelial cells. The presence of squamous epithelial cells indicated oropharyngeal contamination. A simple dilution technique was chosen to compare the isolation of potential pathogens from direct cultures and from dilutions of sputa (10^{-7} per ml original sputum). The dilution technique permitted easier reading of sputum cultures and avoided the possible over-reporting of enterobacteria and *Pseudomonas aeruginosa*, which were frequently found on direct examination. Enterobacteria and *Pseudomonas aeruginosa* were more likely to be isolated from sputa moderately or heavily contaminated with squamous epithelial cells.

The use of counter-current immunoelectrophoresis (CIE) and co-agglutination tests to detect pneumococcal antigen in sputa is described. The presence of antigen in sputum was a more reliable index of lower respiratory tract infection than a positive culture. Co-agglutination tests were simpler to perform and used smaller amounts of expensive antiserum than CIE.

Sputa are contaminated to a greater or lesser degree by organisms from the mouth and pharynx. As some pathogens in the lower respiratory tract, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, are also indigenous to the oral cavity (Brumfitt *et al.*, 1957) and others, such as enterobacteria and *Pseudomonas aeruginosa*, rapidly colonise the upper respiratory tracts of hospital patients receiving antibiotics (Percival and Roberts, 1971), the results of sputum cultures can be very misleading. More accurate methods of sampling the contents of the lower respiratory tract have been described, such as transtracheal aspiration or bronchoscopy (Hahn and Beaty, 1970; Jordan *et al.*, 1976), but these are not generally acceptable as a routine.

The laboratory examination of sputum can be improved in several ways. Firstly, microscopic examination can be used to evaluate the quality of each specimen (Bartlett, 1974; Murray and Washington, 1975). Gram films are examined for the presence of squamous epithelial cells, which strongly suggest

oropharyngeal contamination, and for leucocytes and potential bacterial pathogens as indicators of infection. In this way it is possible to reject specimens with gross oropharyngeal contamination and little or no evidence of lower respiratory tract infection.

Secondly, as infecting organisms are usually present in larger numbers than contaminants from the upper respiratory tract, counts can be used to assess the likely importance of any bacteria present (Dixon and Miller, 1965; Pirtle *et al.*, 1969).

A third improvement is the rapid detection of pneumococcal antigen in sputa by counter-current immunoelectrophoresis (Verhoef and Jones, 1974; El-Refaie and Dulake, 1975; Spencer and Savage, 1976). The presence of antigen appears to be a more reliable index of lower respiratory tract infection than a positive culture, although the cost of the antiserum required may make the test unacceptable to some laboratories.

In this study we have re-evaluated some of the methods for the bacteriological examination of sputum and have assessed the influence of oropharyngeal contamination on the results of sputum culture. We have also modified the slide co-agglutination test described by Kronvall (1973) for typing pneumococci to detect pneumococcal antigen in sputa.

Material and methods

SPECIMENS

Sputum was collected in screw-capped containers (Sterile Universal Container, Sterilin Ltd) by either a nurse or a physiotherapist. As freshly collected sputa (received within one hour of expectoration) were rarely obtained, both microscopic and cultural examinations were routinely performed on homogenised specimens.

HOMOGENISATION OF SPUTUM

All sputa were processed in an exhaust protective cabinet. A few glass beads (2.5-3.5 mm) and an equal volume of 2% (w/v) N-acetyl-L-cysteine (NAC) were added to each specimen. The NAC solution was freshly prepared each day by dissolving 2 g NAC in 13 ml 1N NaOH and diluting to a final volume of 100 ml with PBS (Dulbecco A). The pH of the solution was adjusted to 7.3 if necessary. The caps on the universal containers were securely tightened, the NAC-sputum mixtures were agitated on a vortex mixer for 10 seconds, allowed to stand at room temperature for 10 minutes, and finally vortex mixed for a further 15 seconds. Homogenised sputa were processed within 30 minutes, although duplicate counts were sometimes made on specimens kept at 4°C for 24 hours.

MICROSCOPIC EXAMINATION OF SPUTUM

Using a 1- μ l disposable loop (Nunc products), a loopful of homogenised sputum was spread over a 1 cm² area on a glass slide and a Gram-stained film was prepared. The films were examined under low-power magnification ($\times 20$ objective) for squamous epithelial cells and under high-power magnification ($\times 50$ objective) for leucocytes and potential pathogens. At least 10 fields were examined at each magnification, and the specimens were graded (1-9, see Table 1) according to the numbers of leucocytes and squamous epithelial cells seen. The cut-off points were: squamous epithelial cells 1-8 (per $\times 20$ objective field) moderate contamination, > 8 heavy contamination; and for leucocytes < 5 (per $\times 50$ objective field) not purulent, 5-15 moderately purulent, > 15 purulent.

DIRECT AND QUANTITATIVE (DILUTION) CULTURE OF SPUTUM

Procedure

Direct cultures were prepared by spreading a loopful of homogenised sputum onto 5% horse blood Columbia agar (Oxoid) containing nicotinamide adenine dinucleotide (12.5 mg/l) and haematin (2.5 mg/l). The cultures were incubated overnight

at 37°C in a moist atmosphere containing 4% CO₂ (CO₂ incubator, Grant Instruments Ltd). Quantitative counts were made by adding 0.2 ml homogenised sputum to 0.8 ml of diluent (1 vol single strength nutrient broth + 9 vol PBS). Using a 1- μ l disposable loop, a loopful of this diluted sputum was thoroughly mixed with 1 ml diluent, and from this a further 1 μ l was spread onto 5% horse blood Columbia agar. The plate was incubated overnight at 37°C in a moist atmosphere containing 4% CO₂. After incubation the numbers of different colonial types on direct and dilution plates were counted (each colony on a dilution plate representing 1×10^7 per ml original sputum), and the bacteria were identified by standard methods (Cowan, 1974).

Interpretation of culture results

In order to analyse the results of this study the following bacteria were considered potential pathogens:

Group I *H. influenzae*, *Strep. pneumoniae*, *Staphylococcus aureus*, and *Neisseria meningitidis*.

Group II Enterobacteria, *Ps. aeruginosa*, and *Pasteurella multocida*.

These bacteria were considered numerically significant if they grew at 10^{-7} dilution or if the growth on direct plates was equal to or greater than that of any normal flora present (*ie*, viridans streptococci, commensal neisseria, diphtheroids, and *Staphylococcus albus*).

Replication of dilution counting technique

Homogenised sputum was divided into 10 portions and quantitative counts were made on each sample as previously described. Counts were paired randomly, and the probability of the difference of the pairs being significant ($P \leq 0.05$) was calculated by Student's *t* test.

DETECTION OF PNEUMOCOCCAL ANTIGEN IN SPUTUM

Diagnostic Pneumococcus sera (Omniserum and type 3 antiserum) were purchased from Statens Serum Institut, Copenhagen, Denmark. The sera were stored at 4°C until used.

Counter-current immunoelectrophoresis (CIE)

This was performed on homogenised sputa according to the method described by El-Refaie and Dulake (1975) using veronal-acetate buffer (pH 6.5).

Co-agglutination tests

A stabilised suspension of *Staph. aureus* (Cowan I strain, NCTC No. 8530) was prepared as described by Tebbutt *et al.* (1976), and the staphylococci were coated with pneumococcal antibody (Omniserum or

type 3 antiserum) as described by Kronvall (1973). For each test about 1 ml of homogenised sputum was boiled for 10 minutes and either allowed to stand at room temperature for 5 minutes or centrifuged at 1100 g for 5 minutes. A 2- μ l loopful of the supernatant fluid was mixed with a loopful of antibody-coated staphylococci on a microscope slide, the slide was rocked continuously for about 2 minutes, and the presence or absence of agglutination was recorded.

Results

In this study 17% of specimens received by the laboratory were saliva. Their macroscopic appearance correlated well with the presence of squamous epithelial cells seen on microscopy (81% of saliva specimens). The microscopic examination of 957 sputa using the screening procedure described (see Methods) is shown in Table 1. Purulent or moderately purulent specimens accounted for 58% of sputa received, and 48% of specimens were moderately or heavily contaminated by squamous epithelial cells.

The method of preparing sputum dilutions was reproducible, and statistical analysis (see Methods) revealed no significant differences between bacterial counts from sample pairs of sputa at 10^{-7} dilution. With the exception of sputa heavily contaminated by squamous epithelial cells, specimens yielding no potential pathogens on direct examination often showed no growth at 10^{-7} dilution (75% of dilution plates). The presence of squamous epithelial cells was clearly associated with large numbers of bacteria from the oropharynx, and only 22% of sputa in these grades (3, 6, and 9, see Table 1) showed no growth at 10^{-7} dilution.

A total of 378 specimens (39%) yielded potential pathogens which were considered numerically significant (see Methods). The majority were isolated

from purulent or moderately purulent sputa (282/554 specimens; grades 1 to 6, Table 1). Potential pathogens isolated from non-purulent sputa (96/403 specimens; grades 7, 8, and 9) were predominantly enterobacteria or *Ps. aeruginosa* (see below). On dilution plates yielding potential pathogens, the mean number of colony types was 1.3 and the mean number of colonies was 180 per plate. Contamination of sputa by upper respiratory tract flora did not significantly influence the numbers of colony types or colonies of potential pathogens isolated at 10^{-7} dilution (mean 1.3 and 144 per plate respectively).

In Fig. 1, the isolations of *H. influenzae*, pneumococci, *Staph. aureus*, and meningococci (group I pathogens) are compared with the numbers of squamous epithelial cells and leucocytes observed by microscopy. The pathogens were predominantly

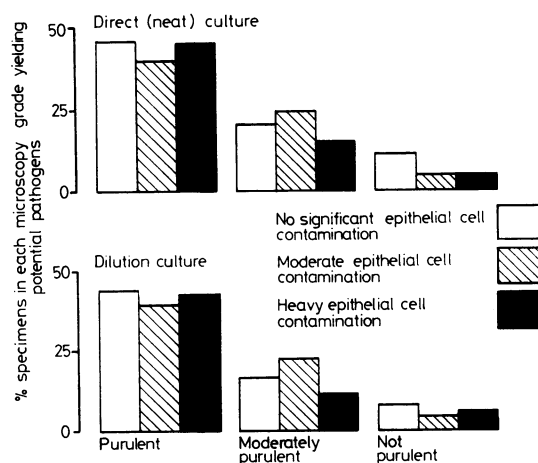


Fig. 1 Relationship between presence of leucocytes and squamous epithelial cells (see Methods) and isolation of group I pathogens from neat and 10^{-7} dilutions of sputa.

Table 1 Microscopic examination of sputa—classification of grades and specimens examined

Grade	Microscopy*		Sputa received		No. of specimens yielding potential pathogens
	Squamous epithelial cell contamination	Leucocytes	No. in each grade	% of total 957 in each grade	
1	Not significant	Purulent	216	23	124
2	Moderate	Purulent	86	9	56
3	Heavy	Purulent	31	3	21
4	Not significant	Moderately purulent	118	12	37
5	Moderate	Moderately purulent	73	8	33
6	Heavy	Moderately purulent	30	3	11
7	Not significant	Not purulent	168	18	42
8	Moderate	Not purulent	162	17	35
9	Heavy	Not purulent	73	8	19
Totals			957		378

*Squamous epithelial cells (per $\times 20$ objective field): 1-8 moderate contamination, > 8 heavy contamination. Leucocytes (per $\times 50$ objective field): < 5 not purulent, 5-15 moderately purulent, > 15 purulent.

isolated from purulent or moderately purulent sputa (191/228, 84%) rather than from non-purulent sputa. Although fewer pathogens (18/228, 8%) were isolated from the heavily contaminated grades (3 and 6; Table 1) this was due to the smaller numbers of specimens in these grades (31 and 30 respectively) rather than to a decrease in the isolation rate from sputa heavily contaminated with material from the oropharynx (Fig. 1). Although similar numbers of group I pathogens were found on direct and dilution plates (228 and 197 pathogens respectively) the pathogens were usually isolated in pure culture on dilution plates and were therefore more easily identified. Sometimes potential pathogens were partly overgrown by upper respiratory tract flora on direct plates or were obscured by the spreading growth of *Proteus* species. In these cases estimation of the numbers of potential pathogens present and the interpretation of their likely significance would be particularly difficult using direct examination alone.

Group II pathogens (predominantly enterobacteria and *Ps. aeruginosa*) were more often isolated on direct than on dilution plates (199 compared with 40 isolations). Growth on direct examination was often associated with the presence of squamous epithelial cells and was not necessarily related to the purulence of the specimens (Fig. 2).

In the co-agglutination test, untreated sputa strongly agglutinated staphylococci not coated with pneumococcal antibody. These non-specific reactions were removed by autoclaving or boiling sputa before examination (see Methods). When the levels of pneumococcal capsular antigen were compared in heated and unheated sputa by rocket (Laurell) immunoelectrophoresis, no decrease in the amount of antigen present was detected after heating.

The detection of pneumococcal antigen in 490 purulent or moderately purulent sputa by CIE and co-agglutination is compared in Table 2. The difference between the results of the two methods was small, and antigen was detected in 94 specimens by co-agglutination and in 83 specimens by CIE. The methods failed to distinguish pneumonia from other

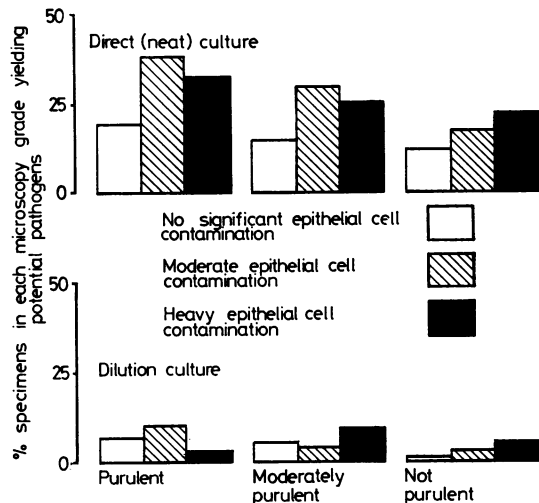


Fig. 2 Relationship between presence of leucocytes and squamous epithelial cells (see Methods) and isolation of group II pathogens from neat and 10^{-7} dilutions of sputa.

chest infections caused by pneumococci. Antigen was detected in about 4% of specimens from which pneumococci were not isolated. The presence of antigen in these specimens correlated well with the presence of antibiotics, or the finding of pneumococci in the Gram film, or both (75% of antigen-positive but culture-negative specimens). With one exception (co-agglutination between type 3 pneumococcal reagent and an *Escherichia coli* isolate), CIE and co-agglutination methods were specific for detecting pneumococcal antigen. Examination of another 300 sputa has further confirmed the specificity of the co-agglutination test for detecting pneumococcal infection.

In some tests the detection of pneumococcal antigen in sputa from which pneumococci had been cultured was compared to the numbers of leucocytes present. Antigen was detected in 85% (78/92) moderately purulent or purulent specimens whereas

Table 2 Detection of pneumococcal antigen in moderately purulent and purulent sputa by CIE and co-agglutination

Diagnosis	No. examined	Culture positive		Culture negative	
		CIE	Co-agglutination	CIE	Co-agglutination
Lobar pneumonia	27	9	9	3	3
Bronchopneumonia	25	4	6	0	1
Acute exacerbation of chronic lung disease	69	7	6	3	3
Postoperative infection	85	16	18	4	6
Chest infection (not specified)	190	28	30	5	6
No relevant information	94	3	5	1	1
Totals	490	67	74	16	20

only 33% (6/18) non-purulent culture-positive sputa had detectable levels of antigen.

Discussion

The experiments described here form part of an investigation into the clinical significance of bacteria isolated from sputum. We have described laboratory techniques for evaluating the quality of sputum and have tried to assess the likely importance of potential pathogens isolated. The techniques are relatively simple to perform and have proved sufficiently accurate for routine examination. Several conclusions can be drawn from the results.

Firstly, we have confirmed that microscopic examination is a valuable guide for determining whether sputum is acceptable for culture. We have obtained potentially misleading information from cultures of non-purulent sputa, particularly those moderately or heavily contaminated by squamous epithelial cells (see Fig. 2). Thus we are considering the rejection of non-purulent specimens with a request for a further sputum. However, difficulties arise with specimens from patients unable to mount an immune response or which would be difficult or hazardous to repeat (for example, from children and cardiac or mechanically ventilated patients). These should not be rejected without first consulting the clinician.

Secondly, microscopic examination showed that about 50% of sputa tested were moderately or heavily contaminated by upper respiratory tract flora. Murray and Washington (1975) considered all heavily contaminated sputa unacceptable for culture. The results of our experiments suggest that culture of moderately purulent or purulent sputa containing numerous squamous epithelial cells is justified. However, the degree of oropharyngeal contamination should be borne in mind when reporting enterobacteria or *Ps. aeruginosa*.

Thirdly, we have some evidence of the probable significance of the potential pathogens isolated. We found that a heavy growth of pneumococcus or *H. influenzae* or both was closely associated with a purulent or moderately purulent sputum. This result complements other evidence that large numbers of these organisms are often associated with radiological and clinical signs of chest infection (Thorsteinsson *et al.*, 1975; Wilkinson *et al.*, 1977). In contrast, the isolation of enterobacteria and *Ps. aeruginosa*, particularly on direct examination, was often associated with oropharyngeal contamination and not necessarily related to purulence. Similar findings have been reported by Percival and Roberts (1971). Although the possibility that enterobacteria and *Ps. aeruginosa* can cause severe chest infections

is recognised (Stevens *et al.*, 1974), over-reporting of these organisms must be avoided. We, like others (Percival and Roberts, 1971), found microscopic evaluation and quantitation of the numbers of coliforms present helpful in determining their likely significance in a sputum.

Fourthly, our results emphasise the value of CIE and co-agglutination tests for the rapid detection of pneumococcal chest infections. We found the co-agglutination test used considerably less antiserum and was simpler to perform than CIE. These advantages are partly off-set by the time taken to prepare the antibody-coated staphylococcal reagent.

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