

Evaluation of anaerobic culture and effect of culture medium supplementation with factor V on colonial morphology and efficacy of isolation of *Streptococcus pneumoniae* from sputum

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SUMMARY The use of anaerobic incubation for the culture of *Streptococcus pneumoniae* from sputum was compared with incubation in carbon dioxide in air. A coagglutination test for pneumococcal antigen was used as an index of the number of specimens containing pneumococci. A total of 334 specimens were examined. There was evidence of pneumococcal colonisation by culture or coagglutination, or both, in 48 (14.37%), of which 41 (12.27%) yielded *S pneumoniae* on culture. Anaerobic incubation was better than incubation in carbon dioxide in air for the primary culture of *S pneumoniae* from sputum. Primary isolation of *S pneumoniae* was achieved in 11 of the 41 strains (26.82%) by anaerobic incubation alone, by incubation only in carbon dioxide in air in one strain (2.43%), and by both anaerobic incubation and incubation in carbon dioxide in air in 29 strains (70.73%). Anaerobic incubation gave large moist or mucoid colonies that were easy to recognise, but it suppressed the typical draughtsman colony of *S pneumoniae*. The factor V supplement routinely used in our medium also inhibited the formation of draughtsman colonies.

It is suggested that draughtsman colonies occur because of a relative lack of the coenzyme nicotinamide adenine dinucleotide (factor V), which is required as a reducing agent in aspartate and glutamate metabolism. This nutritional deficiency may lead to bacterial cell wall defect and hence to the autolysis which gives the typical draughtsman colony.

It may be difficult to isolate *Streptococcus pneumoniae* from sputum even in bacteraemic pneumococcal pneumonia.¹ The problem may be due to spontaneous bacterial autolysis, or to the use of a culture medium that is nutritionally unsatisfactory or insufficiently reduced.²

Our main object was to compare anaerobic culture of sputum specimens for the isolation of *S pneumoniae* with culture in carbon dioxide in air, using a coagglutination test for pneumococcal antigen as an index of the total number of specimens containing pneumococci. We also made a limited assessment of the influence of the culture medium used on the isolation of *S pneumoniae* and we reviewed the pneumococcal serotypes of the strains isolated to determine whether the serotype had any influence on the preferred atmosphere of incubation.

Material and methods

Studies were made of 334 specimens of sputum. Specimens were inspected on receipt at the laboratory and samples of saliva and clear mucus were rejected. Only mucopurulent and purulent specimens were retained for further examination.

CULTURE METHODS

Cultures were prepared before the coagglutination test for pneumococcal antigen was done. The specimen material used for culture was examined without homogenisation. The medium comprised plates of 5-7% horse blood with Oxoid No 2 agar base, supplemented with 20 µg/ml factor V (β nicotinamide adenine dinucleotide, Boehringer Corporation, London). The plates were 90 mm in diameter, and the volume of medium in each plate 20 ml.

INOCULATION PROCEDURE

Two plates were inoculated from a purulent portion of sputum. Specimens were plated out to give single colonies, and an Optochin disc (ethylhydrocuprein hydrochloride, Oxoid) was placed on the primary inoculum streak of each plate.

CONDITIONS OF INCUBATION

One plate was incubated in an atmosphere of 10% carbon dioxide in air. The other plate was incubated anaerobically in an atmosphere of 10% carbon dioxide with 90% hydrogen. Plates were incubated at 37°C overnight and then examined.

IDENTIFICATION OF *S PNEUMONIAE*

Colonies were provisionally identified as *Spneumoniae* if they were α -haemolytic, sensitive to Optochin with a minimum inhibition zone radius of 5 mm, and had colonies which were either "draughtsman" shaped or mucoid.

An initial confirmation was made by the bile solubility test, using the direct method of Hawn and Beebe.³ All colonies provisionally identified as *Spneumoniae* were subcultured for purity and the Optochin sensitivity was retested. Pneumococci isolated in anaerobic culture only were subcultured for purity and a further check of Optochin sensitivity on duplicate plates was made, one incubated anaerobically and one in an atmosphere of 10% carbon dioxide in air. This was done as a final check on colonial morphology, identity, and preferred atmosphere of incubation.

TESTS ON THE INFLUENCE OF THE CULTURE MEDIUM ON THE RECOVERY AND RECOGNITION OF *S PNEUMONIAE**Effect of factor V supplement*

To check that our results were not due to the use of factor V supplemented culture medium we compared the results given by our medium with and without the factor V supplement.

Comparison with culture media used by other laboratories

This was done in case a local vagary in culture medium preparation was responsible for our results. We obtained samples of the culture medium plates

used by two other laboratories (laboratory I and laboratory II). These were tested to compare the colonial appearance given by *S pneumoniae* with that on a similar medium prepared in our laboratory (laboratory III). Laboratories I and II both used a medium of 5-7% horse blood with Columbia agar base (Oxoid). As neither laboratory I nor laboratory II media were supplemented with factor V they were compared with our medium of 5-7% horse blood with Oxoid No 2 agar base made without the factor V supplement.

PROCEDURE FOR TESTS ON DIFFERENT CULTURE MEDIUM

Pure cultures of 19 freshly isolated strains of *Spneumoniae* were used as test strains. Each strain was cultured on two plates of each type of blood agar medium. One set of plates was incubated in 10% carbon dioxide in air and the other set anaerobically in an atmosphere of 10% carbon dioxide with 90% hydrogen.

The coagglutination test (Inverclyde Biologicals, Bellshill, Scotland) was carried out as recommended by the manufacturer. Specimens were homogenised for the coagglutination test using Sputolysin (Oxoid).

SEROTYPING OF *S PNEUMONIAE* STRAINS

Strains were submitted to a reference laboratory where serotyping was performed by a coagglutination method.

Results

Spneumoniae was detected by culture or coagglutination or by both methods in 48 (14.37%) of the 334 specimens examined (table 1).

Anaerobic incubation was superior to incubation in 10% carbon dioxide in air for the detection of *Spneumoniae* by culture.

Of the 41 strains of *Spneumoniae* isolated, 11 (26.82%) were found only in anaerobic culture on primary isolation. On subculture of these strains, two (4.88%) grew only in anaerobic culture. The colony type found on anaerobic incubation was large, shiny, and moist, or mucoid with a viscid consistency. No draughtsman colonies were formed. Of the 41 strains of *Spneumoniae*, one (2.43%) grew only in carbon dioxide in air on primary culture.

Table 1 Comparison of aerobic and anaerobic culture on factor V supplemented blood agar with coagglutination test for pneumococcal antigen on 48 specimens of sputum

Method	No of specimens tested	No of specimens positive (%)	No of specimens negative (%)
Coagglutination test for pneumococcal antigen	47	40 (85.10)	7 (14.89)
Culture in 10% carbon dioxide in air	48	30 (62.50)	18 (37.50)
Culture in 10% carbon dioxide plus 90% hydrogen	48	40 (83.33)	8 (16.66)

Table 2 Morphology of *S pneumoniae* colonies on blood agar with and without added factor V incubated aerobically and anaerobically

Colony type	Blood agar not supplemented with factor V		Blood agar supplemented with factor V	
	10% carbon dioxide in air	10% carbon dioxide plus 90% hydrogen	10% carbon dioxide in air	10% carbon dioxide plus 90% hydrogen
Large, moist, or mucoid	1	15	2	18
Draughtsman	11	0	0	0
Pinpoint	5	1	14	0
No growth	2	3	3	1
Total	19	19	19	19

INFLUENCE OF THE CULTURE MEDIUM ON THE RECOVERY AND RECOGNITION OF *S PNEUMONIAE*

Table 2 shows the effect of the factor V supplement used in 5–7% horse blood agar plates with Oxoid No 2 agar base. Large, moist, or mucoid colonies were formed on anaerobic incubation on both supplemented and unsupplemented medium. Draughtsman colonies appeared only after incubation in carbon dioxide in air and in the absence of the factor V supplement. Culture on the factor V supplemented medium produced pinpoint α -haemolytic colonies after incubation in carbon dioxide in air. These were morphologically indistinguishable from colonies of viridans streptococci.

All three media from the three laboratories had the same capacity to grow *S pneumoniae* as draughtsman colonies on incubation in carbon dioxide in air and as large, moist, or mucoid colonies on anaerobic incubation.

RESULTS OF COAGGLUTINATION TESTS ON SPUTUM SPECIMENS

The coagglutination test was performed on 47 of the 48 specimens in which *S pneumoniae* was detected. Both culture and coagglutination were positive in 32 of the 47 specimens (68.08%), eight specimens (17.02%) were culture positive but coagglutination negative; and seven (14.89%) were coagglutination positive but culture negative.

RESULTS OF SEROTYPING

Serotyping by a coagglutination method was performed on 37 strains. No one type predominated. The most common type was type 9, of which there were six strains.

Discussion

The bacteriological diagnosis of pneumococcal pneumoniae is often unsatisfactory. The reason is not necessarily the culture of poor quality specimens. Even unhomogenised sputum specimens can give results equal to those obtained by the culture of

transtracheal aspirates or bronchoscopy specimens if simple bacteriological methods are performed meticulously, especially if the specimen is plated out so as to obtain single colonies.⁴ A more aggressive approach is to use mouse inoculation with sputum from patients with suspected pneumococcal pneumonia. This can considerably improve the isolation of pneumococci from sputum.^{5,6}

The diagnostic difficulty imposed by antecedent antibiotic treatment is well known,^{6–8} and has led to the development of methods for the detection of pneumococcal antigen. Counterimmunoelectrophoresis is the usual method, but it may give false negative results when compared with conventional culture⁹ or conventional culture used in conjunction with the Quellung reaction.¹⁰ The coagglutination method of pneumococcal antigen detection is said to be more sensitive than CIE¹¹ and superior to the use of culture alone, without microscopy of the sputum as a supportive investigation.⁸ It was for this reason that we used a coagglutination test for pneumococcal antigen to screen specimens for the presence of *S pneumoniae*. Of the 47 specimens positive for *S pneumoniae* tested by the coagglutination method, eight (17.02%) grew *S pneumoniae* on culture but gave a negative result by coagglutination. Coagglutination was therefore of limited value as an assessment of the efficiency of our methods.

We found that anaerobic culture was superior to culture in carbon dioxide for the recognition of *S pneumoniae*. There are several reasons for this result. Anaerobic incubation has the advantage that it produces large easily recognisable colonies of *S pneumoniae*.^{12–14} In addition, it suppresses the growth of unwanted contaminant bacteria to some extent.^{13,14} An alternative approach is to eliminate coliform growth by the use of gentamicin in culture plates,¹⁵ but this has the theoretical disadvantage that the growth of *S pneumoniae* may also be inhibited. Lastly, some strains of *S pneumoniae* are non-aerotolerant on primary isolation, because they have difficulty in establishing growth under unfavourable conditions of oxidation-reduction potential.¹⁶ It is said that reduction of the culture medium prevents

the growth inhibitory effect caused by overheating during the preparation of culture media.¹⁷ We isolated 11 strains of this type, two of which grew only in strict anaerobiosis on subculture. Though *Spneumoniae* strains which require carbon dioxide for growth have been described,^{18,19} the existence of strictly anaerobic strains of *Spneumoniae* is debatable. Such organisms may require strict anaerobiosis for primary isolation, but aerobic variants appear with ageing.²⁰

The improved results obtained by anaerobic incubation could not be attributed to the predominance of a non-aerotolerant serotype of *Spneumoniae* among our strains, nor to a local peculiarity in medium preparation. The reason for the improved recognition and recovery of *Spneumoniae* by anaerobic incubation may be that autolysis was prevented. The production of draughtsman colonies was stimulated by incubation in carbon dioxide in air, but inhibited by anaerobic conditions and factor V. The draughtsman colony is an autolysis phenomenon.² This suggests that factor V is required to prevent autolysis, particularly as factor V acts as a coenzyme in bacterial oxidation-reduction reactions.²¹ There are few studies on the growth requirements of *Spneumoniae*, but it seems that glutamine is needed, along with several other growth factors.²² The coenzyme nicotinamide adenine dinucleotide (NAD, factor V) not only has a role in glutamine synthesis, but also acts as a reducing agent in the branch of aspartate metabolism, ultimately concerned with the synthesis of methionine.²³ Methionine, like glutamine, is an essential factor in cell wall biosynthesis.²⁴ This suggests that draughtsman colonies may be a manifestation of cell wall defect and that they appear when the culture medium is inadequately reduced, or if there is a relative deficiency of NAD. This theory explains the superiority of anaerobic incubation for the culture of *Spneumoniae* and the absence of draughtsman colonies under conditions of anaerobic incubation.

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