J Clin Pathol 1986;**39**:871–875

Identifying non-capsulate strains of *Streptococcus pneumoniae* isolated from eyes

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SUMMARY Over six months 10 biochemically and physiologically atypical strains of *Streptococcus pneumoniae* were isolated from eye swabs. Conventional methodology showed that these strains possessed characteristics of both *S pneumoniae* and other α haemolytic streptococci. The use of sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS–PAGE) enabled us to characterise these strains as *S pneumoniae*. Loss of the capsule seemed to be associated with atypical biochemical properties.

Streptococcus pneumoniae is the commonest pathogen of lobar pneumonia and has been implicated in other infections, including meningitis, septicaemia, and conjunctivitis.[1–3] Occasionally, other α haemolytic streptococci (αHS) have been isolated from conjunctival specimens, but their clinical importance remains doubtful.[1 3 4] Shayegani *et al*[5] described outbreaks of conjunctivitis in New York State during which atypical αHS were isolated from 46% of specimens. The strains were submitted to the Center for Disease Control, Atlanta, and the Statens Seruminstitut, Copenhagen, both of which identified the isolates as *S pneumoniae*. If, however, the commonly used identification scheme of Facklam[6] was applied to these strains they became designated *S morbillorum*, and they would therefore be of questionable importance from this site.

Clearly, therefore, it is important for the clinical laboratory to identify correctly isolates of αHS from cases of conjunctivitis to determine whether they are *S pneumoniae*.

Over six months 10 strains of αHS that displayed atypical characteristics of *S pneumoniae* were isolated from eye swabs received by the departments of bacteriology of the Childrens' Hospital, and Royal Hallamshire Hospital, Sheffield. These strains were sensitive to optochin but were insoluble in 10% desoxycholate and biochemically could not be classified as *S pneumoniae*.

To characterise the αHS we subjected them to a range of recognised laboratory techniques, including optochin sensitivity, bile solubility, biochemical identification, coagglutination using pneumococcal omniserum and sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS–PAGE). On the basis of the results obtained we report the identification of these strains as non-capsulate *S pneumoniae*.

Accepted for publication 5 March 1986

Material and methods

BACTERIAL STRAINS

Ten strains of non-capsulate *S pneumoniae* were isolated from eye swabs on 5% chocolatised horse blood agar and 5% horse blood agar incubated aerobically (plus 10% carbon dioxide) and anaerobically (80% nitrogen : 10% carbon dioxide : 10% hydrogen), respectively, at 37°C.

Two capsulate strains of *S pneumoniae* and a representative strain of *S mitis* and *S sanguis* were obtained from clinical specimens. These strains were identified by standard laboratory procedures. Two strains of *Gemella haemolysans* were obtained from l'Institute Pasteur, Paris, via API Laboratory Products.

BIOCHEMICAL IDENTIFICATION

This was performed using API 20 Strep (API Laboratory Products). The system was inoculated, incubated, and interpreted according to the manufacturer's recommendations. Identification was determined using the Apilab computer program.

OPTOCHIN SENSITIVITY (DISC DIFFUSION)

The test organisms and control strains were suspended in physiological saline to an optical density equivalent to McFarland 0·5 standard. Five per cent horse blood agar plates were flooded with these sus-

pensions and allowed to dry. Optochin discs (5 μg) were applied to the surface and the plates incubated at 37°C aerobically. After incubation the diameters of any zones of inhibition were recorded.

OPTOCHIN SENSITIVITY (MINIMUM INHIBITORY CONCENTRATION)

Minimum inhibitory concentration (MIC) to optochin was determined by incorporating a range of dilutions of ethyl hydrocupreine hydrochloride (Sigma) into 5% horse blood agar plates. Saline suspensions of the organisms, equivalent to McFarland 0·5 standard were spotted on to the plates using a multipoint inoculator (Diamed). After overnight aerobic incubation at 37°C the MIC to each strain was interpreted as the highest dilution of optochin that inhibited visible growth.

BILE SOLUBILITY

A spot test was used with 10% sodium desoxycholate (BDH) substituted for 2%.[7] Positive reactions were recorded as the disappearance of the colony within five minutes.

COAGGLUTINATION WITH PNEUMOCOCCAL OMNISERUM

Staphylococcal protein A was prepared, as described previously.[8] The pneumococcal suspension was prepared by reacting 0·05 ml of pneumococcal omniserum (Statens Seruminstitut, Copenhagen) with 1·25 ml of 4% staphylococcal protein A. Non-immune rabbit serum was substituted for pneumococcal omniserum to serve as a negative control reagent. Test colonies were picked from agar plates with a nichrome wire and emulsified in 0·01 ml of pneumonococcal suspension and negative control reagent on a microscope slide and mixed. A positive reaction produced agglutination within 30 seconds using pneumococcal suspension. No agglutination occurred with negative control reagent.

SDS–PAGE

Bacterial strains were cultured in 20 ml volumes of medium containing 15 g/l tryptone (Oxoid); 5 g/l yeast extract (Lab M); 0·33 g/l cysteine (BDH); 5 g/l glucose, and 2 g/l sodium carbonate and incubated overnight at 37°C. Cells were harvested by centrifugation at 1500 *g* for 30 minutes and washed twice in distilled water before being extracted by incubation in an equal volume of SDS sample buffer[8] for two hours at room temperature. The cell debris was removed by centrifugation at 15 000 *g* for 10 minutes, and samples of the supernatant were examined by PAGE.

Electrophoresis was performed in the presence of SDS using the buffer system of Laemmli[9] with 9% (w/v) acrylamide in the separating gel. Gels were run at a constant current of 30 mA until the bromophenol blue tracker dye reached the bottom, after which they were fixed and stained in one step by immersion in 0·2% (w/v) Comassie brilliant blue (PAGE blue 83; BDH) in acetic acid and ethanol and water (10% : 45%:45%). Destaining was effected in a similar solvent mixture using methanol instead of ethanol.

Results

SENSITIVITY TO OPTOCHIN (DISC DIFFUSION)

All test strains and capsulate *S pneumoniae* were inhibited by optochin, even after several subcultures. The single strain of *S mitis* showed a zone of inhibition on primary isolation, but after five subcultures it grew up to the edge of the disc. The initial results are summarised in Table 1.

SENSITIVITY TO OPTOCHIN (MIC)

All test strains and capsulate *S pneumoniae* showed equal sensitivity to optochin. Unfortunately, MIC determination to the single *S mitis* strain was not carried out at the time of primary isolation. All organisms were tested in a single batch after several subcultures. Table 2 summarises the results.

BILE SOLUBILITY

All test strains remained intact after five minutes' exposure to 10% sodium desoxycholate. They were therefore designated bile insoluble.

COAGGLUTINATION WITH PNEUMOCOCCAL OMNISERUM

All test strains failed to agglutinate in staphylococcal protein A, indicating absence of detectable capsular polysaccharide.

BIOCHEMICAL IDENTIFICATION

Strains were identified using API 20 Strep. Table 3

Table 1 *Sensitivity to optochin of test and control strains using disc diffusion method*

Strain reference	*Zone diameter (mm)*
2022 Test strain	18
212 Test strain	22
13 Test strain	22
15 Test strain	19
288 Test strain	21
279 Test strain	21
6 Test strain	22
36 Test strain	20
216 Test strain	22
5 Test strain	22
2076 *S mitis*	18
1995 *S sanguis*	No zone
2121 *S pneumoniae*	22
2216 *S pneumoniae*	20

Table 2 *MIC of optochin to test and control strains*

Strain reference	*Concentration of optochin (mg/l)*						
	48	*24*	*12*	*6*	*3*	*1·5*	*0·75*
2022 Test strain	–	–	–	–	–	–	+
212 Test strain	–	–	–	–	–	–	+
13 Test strain	–	–	–	–	–	–	+
15 Test strain	–	–	–	–	–	–	+
288 Test strain	–	–	–	–	–	–	+
279 Test strain	–	–	–	–	–	–	+
6 Test strain	–	–	–	–	–	–	+
36 Test strain	–	–	–	–	–	–	+
216 Test strain	–	–	–	–	–	–	+
5 Test strain	–	–	–	–	–	–	+
2076 *S mitis*	–	+	+	+	+	+	+
1995 *S sanguis*	+	+	+	+	+	+	+
2121 *S pneumoniae*	–	–	–	–	–	–	+
2216 *S pneumoniae*	–	–	–	–	–	–	+

– = No visible growth; + = visible growth.

summarises the numerical profiles and respective probable identities.

The two test strains designated *G haemolysans* were differentiated from the other test organisms by their ability to ferment mannitol and sorbitol. All test strains, however, differed from the two capsulate *S pneumoniae* by failing to produce α galactosidase and being unable to ferment lactose, trehalose, and raffinose, even after 24 hours' incubation.

SDS–PAGE

At least 40 protein bands in the cell extracts were resolved using 9% SDS–PAGE; the Figure shows a representative gel. Tracks 1 and 2 of the gel contained extracts of separate *G haemolysans* strains which differ in band pattern from each other and from the two capsulate pneumococcal strains (tracks 3 and 4). The remaining tracks on the gel (5–13) contained extracts of nine test strains. There was remarkable homogeneity between all of the test strains and the capsulated strains of pneumococci. Of particular note was the major band (arrow figure) that was present in all extracts, except those of *G haemolysans*, emphasising the difference between this species and our test organisms.

Table 3 *Biochemical identification of test strains using API 20 Strep*

Strain reference	*API 20 Strep profile*	*Probable identity*
2022 Test strain	0040001	*S morbillorum*
212 Test strain	0040011	*S morbillorum*
13 Test strain	0040001	*S morbillorum*
15 Test strain	0040001	*S morbillorum*
288 Test strain	0040001	*S morbillorum*
279 Test strain	0040311	*G haemolysans*
6 Test strain	0040311	*G haemolysans*
36 Test strain	0040001	*S morbillorum*
216 Test strain	0040001	*S morbillorum*
5 Test strain	0040001	*S morbillorum*
2121 *S pneumoniae*	0251451	*S pneumoniae*
2216 *S pneumoniae*	0240451	*S pneumoniae*

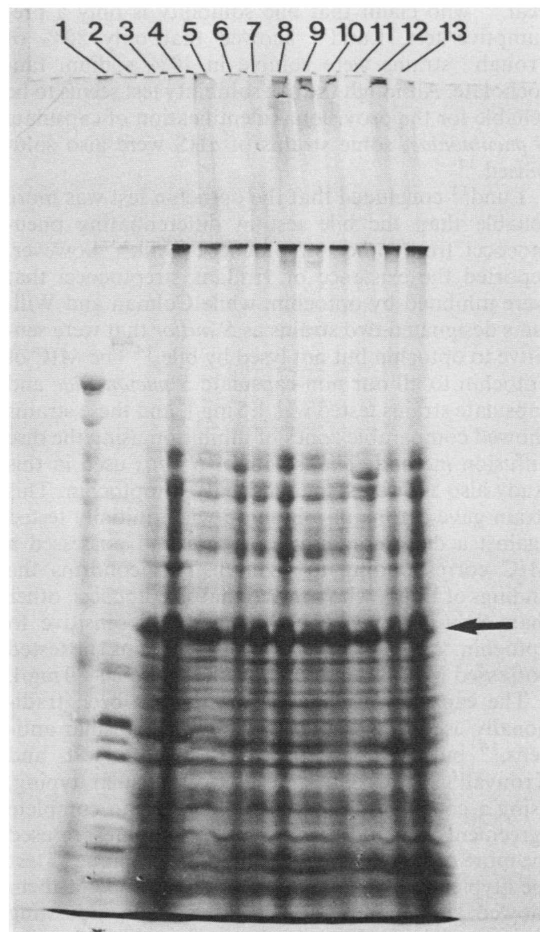

Figure *SDS–PAGE of test and control strains. Tracks 1/2 Gemella haemolysans; tracks 3/4 capsulate S pneumoniae; tracks 5–13 test strains.*

Discussion

Reports describing the isolation of αHS, other than *S pneumoniae*, from eye cultures vary in their conclusions. Perkins *et al*[1] isolated αHS from 26% of cultures in a normal control group compared with 17·9% in an infected group. Results from Prentice *et al*,[2] however, studying neonates, showed isolation rates of 2·9% and 16% in normal and infected groups, respectively. Similar findings were made by Gigliotti *et al*.[3] The clinical importance of non-pneumococcal αHS therefore remains in doubt, but the clinical importance of *S pneumoniae* has been well documented, and so reliable identification is important. Traditionally, this is performed by proving sensitivity to optochin or solubility in bile.

Our results confirm the conclusions of Finegold et al,[10] who claim that bile solubility is only a presumptive test. Lund[11] showed that only 80% of "rough" strains were soluble in 10% sodium taurocholate. Although the bile solubility test seems to be reliable for the provisional identification of capsulate *S pneumoniae*, some strains of αHS were also solubilised.[12]

Lund[11] concluded that the optochin test was more reliable than the bile test in differentiating pneumococci from other streptococci. Hall,[13] however, reported the existence of viridans streptococci that were inhibited by optochin, while Colman and Williams designated two strains as *S mitior* that were sensitive to optochin but not lysed by bile.[14] The MIC of optochin to all our non-capsulate *S pneumoniae* and capsulate strains tested was 1·5 mg/l, and these strains showed comparable zones of inhibition using the disc diffusion method. The isolate of *S mitis* used in this study also showed some sensitivity to optochin. This strain gave a zone of inhibition when initially tested against a disc containing optochin and possessed a MIC corresponding to 48 mg/l. This confirms the findings of Lund, who showed that streptococci, other than *S pneumoniae*, could be partially sensitive to optochin.[11] Nineteen per cent of the strains she tested possessed MICs to optochin in the range 20–80 mg/l.

The capsular swelling reaction[15] has been traditionally used to indentify serologically capsular antigens,[16] but it is laborious as a routine test, and Kronvall[17] showed that results of capsular typing, using a coagglutination technique, were in complete agreement with capsular swelling. We therefore used the more convenient coagglutination technique to test the atypical pneumococcal strains, but none of them showed any agglutination in this system, indicating absence of detectable capsular polysaccharide.

It is not normal practice for laboratories in the United Kingdom to identify *S pneumoniae* biochemically, while other αHS are commonly identified using the API 20 Strep system, which has been shown to give reliable results for most strains.[18] The numerical profiles of our test organisms, using API 20 Strep, were all very similar and identified as *S morbillorum* or *G haemolysans*. Facklam[19] suggested that these organisms should be designated as a single species, but interestingly, the SDS–PAGE profiles of the two *G haemolysans* strains given to us differed considerably from each other, suggesting that this is, in fact, a heterogeneous group.

We referred representative cultures of our strains to two reference centres in the United Kingdom, and although both confirmed our biochemical and physiological results, they disagreed not only with each other but also with our tentative identification of atypical pneumococci (personal communication).

As conventional techniques gave ambiguous conclusions, we carried out whole cell protein analysis using SDS–PAGE. Although this method is not universally used in clinical laboratories, Whiley *et al*[20] separated 100 strains of oral streptococci into seven clusters, and Hoijmakers *et al*[21] concluded that SDS–PAGE could be used as a sensitive identification method for streptococci. Previous work (data not shown) showed that SDS–PAGE of representative strains of *S mitis*, *S sanguis*, *S salivarius*, and *S milleri* gave protein band patterns distinct from each other and from those of the test organisms. The results shown in the Figure, however, showed a striking similarity between capsulate pneumococci and the test strains, both of which differed considerably from *G haemolysans*.

Over the 6 months in which this study took place no similar bile insoluble, biochemically atypical, non-capsulate pneumococci were isolated from any other body site. It is difficult to assess the clinical importance of such organisms as the only accepted pathogenic factor of *S pneumoniae* is the antiphagocytic role of the capsule. All strains were isolated from swabs of infected eyes, and no other recognised bacterial pathogens were detected.

In accordance with Shayegani *et al*,[5] we believe that tear lysozyme or immunoglobulins may have removed the capsular polysaccharide, but no supporting evidence has been found in published reports concerning loss of capsule being associated with changes in biochemical properties.

Conventional methods for the identification of non-capsulate *S pneumoniae* seem to be of little value, but results obtained using SDS–PAGE are encouraging, although the technique is not in widespread use. Therefore, a method for the reliable biochemical identification of non-capsulate *S pneumoniae* would be advantageous, and work is continuing to this end. We intend to determine whether loss of the pneumococcal polysaccharide capsule can cause a change

in biochemical test results and also to refer the test strains for DNA hybridisation studies.

We are grateful for the supply of α haemolytic streptococci and *S pneumoniae* from colleagues at the Children's Hospital, Sheffield; District General Hospital, Barnsley; Victoria Hospital, Worksop; and the Public Health Laboratory, Sheffield, without whose help this study could not have been undertaken.

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