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A comparison of blood agar supplemented with NAD with plain blood agar and chocolate blood agar in the isolation of *Streptococcus pneumoniae* and *Haemophilus influenzae* from sputum

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Streptococcus pneumoniae grows well and generally exhibits typical morphology on Columbia blood agar, whereas *Haemophilus influenzae* requires a more complex medium to meet its growth requirements – usually chocolate blood agar – on which *S. pneumoniae* is less easily recognisable. Therefore, a single medium that produces typical morphology of *S. pneumoniae* and facilitates the growth of *H. influenzae* would have considerable potential advantages. It has been claimed that blood agar supplemented with nicotinamide adenine dinucleotide (NAD) is such a medium. However, despite its routine use in several large diagnostic laboratories its performance has never been properly evaluated. In the present study, 1724 sputum samples were examined in four laboratories. The isolation rates of *H. influenzae* and *S. pneumoniae* on NAD-supplemented blood agar (SBA) were compared with those on a two-plate combination of plain blood (BA) and chocolate blood agar (CBA). The two-plate combination performed significantly better for both organisms; isolation rates for *H. influenzae* were increased from 8.16% on SBA to 11.07% on BA plus CBA and for *S. pneumoniae* from 4.18% to 4.68%. Isolation rates were also compared after incubation for 24 and 48 h. With the two-plate combination, isolation rates for *H. influenzae* and *S. pneumoniae* were increased by 0.98% and 0.16%, respectively, and for SBA by 0.57% and 0.32% after 48 h. However, despite this increase, SBA still performed less well than the two-plate combination.

Introduction

The growth requirement of *Haemophilus influenzae* for haem compounds and nicotinamide adenine dinucleotide (NAD) [1, 2] has led to the widespread use of chocolate blood agar (CBA) for its isolation from sputum samples. Haem compounds are abundant in blood and the heating step in the 'chocolating' process both liberates NAD from erythrocytes and inactivates serum NADases [3], producing a medium rich in both growth factors. However, the other major respiratory pathogen, *Streptococcus pneumoniae* – which grows

well on plain blood agar (BA) and generally exhibits colonies of typical morphology – may be difficult to recognise on CBA, necessitating the use of both BA and CBA plates for the routine examination of sputum.

Therefore, it has been the practice, in several laboratories, to avoid the use of two plates by supplementing blood agar with NAD, thereby theoretically providing conditions suitable for the isolation of both major respiratory pathogens, with typical morphological appearances as well as reduced costs. Although NAD-supplemented blood agar (SBA) has been used as a primary isolation medium in at least one previous study [4], no published work was found that determined its comparative efficacy.

An evaluation of these media was undertaken in four laboratories, adopting a standardised protocol. During this study, data were also collected to determine the

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optimal incubation time for the primary isolation of *H. influenzae* and *S. pneumoniae*, because opinions vary as to whether incubation for 24 h is sufficient, but no published work on this subject was found.

Materials and methods

Media

BA was produced by adding defibrinated horse blood to Columbia Agar Base (CM331; Oxoid) to give a concentration of 5%. CBA was made by heating the plain BA at 75°C until chocolate. SBA was BA supplemented with NAD (Merck; ref. no. 42032) 20 mg/L.

All BA and CBA plates were produced in one laboratory and all SBA plates were produced at a separate laboratory to avoid inter-laboratory variations in media. All media were prepared according to standard protocols and subjected to full quality control procedures before distribution. All plates were used within 14 days of preparation on the basis of pre-trial shelf-life testing which showed no decline in recovery rates or colony size over this period.

Samples

In all, 1724 routine sputum samples received from both hospital and general practice were included in the study. Sputum from immunocompromised patients, intensive care patients and patients with cystic fibrosis, bronchial lavage specimens, nasopharyngeal aspirates and pleural fluids were specifically excluded.

Inadequate specimens were identified and rejected on the basis of a ratio of <2:1 pus cells:squamous epithelial cells as determined by low-power microscopy of a gram-stained preparation of the undigested sample.

Pre-treatment of samples

The sample was homogenised by adding an equal volume of dithiothreitol 0.1% w/v and agitating gently for *c.* 10 s. The sample was then incubated at 37°C for 15 min, followed by gentle agitation for *c.* 15 s. The homogenised sample was diluted by inoculating a 10- μ l loopful into 5 ml of sterile distilled water and mixing gently to give a dilution of 1 in 1000.

Media inoculation and incubation

With a new, sterile loop, 10 μ l of the prepared sample were inoculated on to each agar plate and spread to give individual colonies. An optochin 5- μ g disk was placed on to the SBA and BA plates and a bacitracin 10-U disk on to the CBA plate in the region of the original inoculum to facilitate identification. All plates were incubated in air with CO₂ 5% at 36°C $\pm$ 1°C for 40–48 h. SBA plates were incubated in separate racks.

Plate reading

The staff of laboratories previously inexperienced with any of the above media were given *c.* 2 weeks training before the study to ensure familiarity with colonial appearances. BA and CBA plates were read independently from the SBA plates, by a different member of staff, at 24 and 48 h. Data on bacterial growth and potential pathogens picked were recorded at the time of reading and verified daily by a separate, senior member of staff. Bacterial growth was recorded semi-quantitatively on the basis of colony counting, i.e., 1–10 colonies represent 10⁵–10⁶ cfu/ml in the original sputum; 10–100 colonies represent 10⁶–10⁷ cfu/ml and >100 colonies represent >10⁷ cfu/ml.

Identification

S. pneumoniae was identified on the basis of typical appearance and optochin sensitivity. Typical appearance was defined as raised circular colonies, *c.* 1 mm in diameter, often having a depressed centre due to autolysis – ‘draughtsman’ colonies – and producing α -haemolysis. Capsulate strains may appear watery or mucoid. *H. influenzae* was identified on the basis of colonial morphology and by X and V factor dependence on nutrient agar. A bacitracin disk was used to suppress gram-positive background microflora, thereby facilitating the growth and recognition of *Haemophilus* spp. Isolates from SBA were identified independently of those from BA and CBA.

Statistical methods

Data were collated centrally and entered into an Excel spreadsheet. The discrepancy rates at the four laboratories and the distribution of discrepancies between the methods were analysed by Fisher's 2-sided exact test and the binomial distribution.

Results

Between Nov. 1997 and March 1998, 1724 sputum samples were examined; the four laboratories examined 329, 353, 475 and 567 samples each, respectively. Results are summarised in Tables 1 and 2.

For *S. pneumoniae*, 16 discrepant results were identified, split 13 to 3 in favour of the BA + CBA

Table 1. Comparison of numbers of isolates of *S. pneumoniae* and *H. influenzae* on SBA and BA + CBA

	Isolated on SBA	Not isolated on SBA
<i>S. pneumoniae</i>		
Isolated on BA + CBA	67	13
Not isolated on BA + CBA	3	1641
<i>H. influenzae</i>		
Isolated on BA + CBA	133	52
Not isolated on BA + CBA	4	1535

Table 2. Percentage isolation rates of *S. pneumoniae* and *H. influenzae* on different media

Species	Percentage positivity				Any medium
	SBA	BA	CBA	BA or CBA	
<i>S. pneumoniae</i>	4.18	4.58	4.15	4.68	4.84
<i>H. influenzae</i>	8.16	5.28	10.97	11.07	11.31

combination. The *p* value for this was 0.021, suggesting a higher isolation rate for BA + CBA. There was no statistical evidence of a difference between the laboratories in either the overall discrepancy rate or the pattern of discrepancies.

For *H. influenzae*, 56 discrepancies were split 52 to 4 in favour of BA + CBA, for which the *p* value was <0.0001. No difference was found in the pattern of discrepancies between laboratories, although a significant difference (*p* = 0.002) in overall discrepancy rates was found due to higher rates of discrepancy at two of the testing laboratories.

Data on whether isolation was determined at 24 or 48 h were available for most positive samples from three laboratories. There was no significant difference in the increase from 24 to 48 h between laboratories, so combined results are given in Table 3.

There was an increase in isolation rates at 48 h with all media, although a greater increase was seen with SBA for *S. pneumoniae* (0.32% compared with 0.16% on BA + CBA) and with BA + CBA for *H. influenzae* (0.98% compared with 0.57% for SBA). These differences are not statistically significant.

The upper end of the 95% confidence intervals for the increase in isolation rates at 48 h indicates how large the benefit of incubation for an extra 24 h may actually be – for BA + CBA this value was 0.58% for *S. pneumoniae* and 1.71% for *H. influenzae* and for SBA the values were 0.83% and 1.16% respectively. This would equate to a potential increase over base rates of 12% for *S. pneumoniae* and 21% for *H. influenzae* on BA + CBA; and 21% for *S. pneumoniae* and 15.9% for *H. influenzae* on SBA.

Discussion

Evans *et al.* [5] demonstrated that although most strains of *H. influenzae* require 1–5 mg of NAD/L to

achieve optimal growth, up to 25 mg/L are required for some strains of *H. parainfluenzae*. For this reason, an NAD concentration of 20 mg/L was chosen for BA supplementation in this study, as it should facilitate the growth of *Haemophilus* spp. and allow for a degree of inactivation by serum NADases over time.

The relatively poor performance of SBA in the isolation of *H. influenzae* may be explained by the presence of NADases in the horse blood inactivating NAD added to the media. However, work by Krumwiede and Kuttner [3] would suggest that if NAD is added after blood has been incorporated in a large batch of medium, the dilution effect would minimise NADase activity. Furthermore, during routine shelf-life testing of the media, strains of *H. influenzae* grew equally well on SBA at 21 days as when the plates were fresh.

It has also been suggested that the concentrations of NAD used in the SBA may have had an inhibitory effect on *H. influenzae*. However, in the study by Evans *et al.* [5], concentrations of 25 mg/L had no inhibitory effect and the authors pointed out that *Haemophilus* media based on yeast extracts in the past would have contained as much as 150 mg of NAD/L.

It is possible that NAD may have an inhibitory effect on *S. pneumoniae*, which may explain the rather surprising difference in isolation rates between SBA and BA, although there is no supportive evidence for this, and the differences are so small that they may be a chance finding. Brogan *et al.* [6] described inhibition of the formation of 'draughtsman' colonies by NAD 20 mg/L, believed to be due to the prevention of autolysis, but no influence on growth or isolation rates was observed.

The most likely explanation for the better *H. influenzae* isolation with CBA is that whereas SBA does not contain lysed blood, CBA does, by virtue of the chocolate process, thus allowing the liberation of other growth-enhancing substances, e.g., thiamine and uracil [7].

Although data were available from only three laboratories, further isolations of both pathogens on both media were obtained at 48 h. However, for *S. pneumoniae*, the benefits of extended incubation were less pronounced. For *H. influenzae* on BA + CBA, where the upper end of the 95% confidence interval

Table 3. Comparison of isolation rates on different media at 24 and 48 h

Organism and medium	Positive/total (%)	Time of isolation not recorded	Number of isolates at 24 h	Extra isolates at 48 h	Percentage increase from 24 to 48 h (95% CI)
<i>S. pneumoniae</i> BA + CBA	72/1249 (5.76)	10	60	2	0.16% (0.02, 0.58%)
<i>S. pneumoniae</i> SBA	65/1249 (5.2)	11	50	4	0.32% (0.09, 0.83%)
<i>H. influenzae</i> BA + CBA	140/1249 (11.21)	26	102	12	0.98% (0.51, 1.71%)
<i>H. influenzae</i> SBA	112/1249 (8.97)	14	91	7	0.57% (0.23, 1.16%)

was 1.71%, there appears to be potential for up to 15% more isolates with extended incubation than would otherwise have been detected.

In conclusion, this large multi-centre study shows that SBA performed poorly in the isolation of *H. influenzae* and *S. pneumoniae* compared with the traditional combination of BA + CBA, and may even inhibit the growth of *S. pneumoniae* to some extent. There is no justification for its use in place of BA + CBA.

Incubation of plates for 48 h rather than 24 h resulted in increased isolation rates of *H. influenzae* regardless of media composition, although BA + CBA still performed better than SBA. The isolation rate of *S. pneumoniae* was also improved, but to a lesser extent. On the basis of these results, incubation for 48 h is recommended for the isolation of these two organisms.

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