SHORT ARTICLE

A comparison of the performance of bacitracin-incorporated chocolate blood agar with chocolate blood agar plus a bacitracin disk in the isolation of Haemophilus influenzae from sputum

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The lack of selectivity of chocolate blood agar (CBA), routinely used for the isolation of Haemophilus influenzae, may lead to masking of the growth of H. influenzae due to overgrowth of competing flora. Bacitracin can be used as a selective agent, either incorporated into the medium or applied to the medium in a filter paper. However, neither method has been evaluated or compared in a large study. Sputum samples (1990) were examined in four laboratories and the isolation rates of H. influenzae on chocolate blood agar with bacitracin added to the medium (BCA) and chocolate blood agar (CBA) with a bacitracin disk were compared. A plain blood agar plate was also inoculated to facilitate the isolation of Streptococcus pneumoniae so that its effects on the isolation of H. influenzae could be assessed. No significant difference was found between the isolation rates of H. influenzae on BCA and CBA with a bacitracin disk, although competing flora was greatly reduced and quantity of growth of H. influenzae increased on BCA. The presence of S. pneumoniae did not affect the isolation of H. influenzae in this study.

Introduction

Chocolated blood agar (CBA), produced by heating horse blood agar, is widely used for the isolation of Haemophilus influenzae from clinical samples because it contains essential growth factors, haem compounds and nicotinamide adenine dinucleotide (NAD) [1, 2] in abundance. However, its lack of selectivity may result in the overgrowth of the normal microbial flora from non-sterile sites, obscuring any pathogens present. Bacitracin is a peptide antibiotic which is too toxic for systemic therapeutic use. Its antibacterial spectrum covers most gram-positive bacteria, including most strains of Staphylococcus aureus and Streptococcus pyogenes, as well as Neisseria species, but not H. influenzae. This makes it potentially suitable for use as a selective agent for the isolation of H. influenzae from specimens (e.g., sputum) which may be contaminated with upper respiratory tract flora.

Although published work exists regarding the efficacy of CBA incorporating bacitracin [3–5], similar results may be achieved by placing a bacitracin-containing disk on to the area of the initial inoculum of a CBA plate, allowing suspect colonies to be picked from the zone of inhibition around the disk. This latter option, if equally effective for isolation of H. influenzae, would be more cost-effective as there would be no requirement for a medium formulation specific for respiratory specimens. We are aware of only one previously published direct comparison between the two methods [4]; however, comparative data were available on only 95 mixed respiratory samples and no distinction was made between H. influenzae and other Haemophilus spp. Therefore, the two methods were evaluated in four laboratories with a standardised protocol.

Materials and methods

Media

Blood agar (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 blood agar to 75°C until chcolatised. CBA containing

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bacitracin (BCA) was produced by supplementing CBA with bacitracin (Pro-Labs) 5000 IU/L. All media were produced in a single laboratory to avoid inter-laboratory variation, according to standard protocols and subjected to full quality control procedures before distribution. All plates were used within 14 days of preparation.

Samples
Routine sputum samples (n = 1990) 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 excluded. Inadequate specimens were identified and rejected on the basis of a ratio of polymorphonuclear leucocytes: squamous epithelial cells of < 2:1 as determined by low-power microscopy of a gram-stained preparation of the undigested sample. All samples were processed on the day of receipt.

Processing
Each sputum sample was homogenised by adding an equal volume of dithiothreitol 0.1% w/v and agitating gently for c. 10 s. The dilute sample was incubated at 37°C for 15 min, followed by gentle agitation for c. 15 s. The homogenised sample was diluted by inoculating a 10−1 loopful into 5 ml of sterile distilled water and mixing gently to give a 1 in 105 dilution.

With a new sterile loop, 10 µl of the prepared sample were inoculated on to each agar plate and spread to give individual colonies. A bacitracin disk (10 U) was placed on the CBA plate in the region of the original inoculum. An optochin disk (5 µg) was similarly placed on the BA plate to facilitate identification of 

*S. pneumoniae*. All plates were incubated in air with CO₂ 5% at 36°C ± 1°C for 40–48 h.

Plate reading
BA and CBA plates were examined independently from the BCA plates by a different member of staff at 24 h 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 represented 10²–10³ cfu/ml in the original sputum, 10–100 colonies represented 10⁴–10⁵ cfu/ml and > 100 colonies represented > 10⁶ cfu/ml. A record was made also of whether the bacitracin disk was considered helpful in the identification of 

*H. influenzae* on the BCA plate.

Identification
Isolates of *H. influenzae* on BCA were identified independently of those from CBA.

*H. influenzae* was identified on the basis of colonial morphology and by X and V factor dependence on nutrient agar. *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 with a depressed centre due to autolysis – ‘draughtsman’ colonies – and being α- haemolytic. Capsulate strains appeared watery or mucoid.

Statistical methods
To enable the detection of small differences, the total size of study required was estimated after testing c. 500 samples. After testing, data were collated centrally and entered into an Excel database for analysis. Isolation rates on BCA and CBA were compared by an exact binomial test for paired proportions. Laboratory isolation rates were compared by χ² test.

Results
Between Nov. 1998 and March 1999, 1990 sputum samples were examined; 426, 506, 526 and 532 samples from each of the participating laboratories.

The difference in overall isolation rates for *H. influenzae* was 0.5% in favour of BCA, but this was not statistically significant (p = 0.143) (Table 1). There was no significant difference in the pattern or number of discrepant results between laboratories. However, isolation rates differed significantly across the sites (p = 0.005) (Table 2). This was believed to be due to

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Isolated on BCA</th>
<th>Not isolated on BCA</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated on BCA</td>
<td>251</td>
<td>14</td>
<td>265</td>
</tr>
<tr>
<td>Not isolated on BCA</td>
<td>24</td>
<td>1701</td>
<td>1725</td>
</tr>
<tr>
<td>CBA Totals</td>
<td>275</td>
<td>1715</td>
<td>1990</td>
</tr>
</tbody>
</table>

**Table 1.** Comparison of isolation of *H. influenzae* on BCA and CBA

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>BCA</th>
<th>CBA</th>
<th>Either medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9.89</td>
<td>8.94</td>
<td>10.27</td>
</tr>
<tr>
<td>B</td>
<td>13.85</td>
<td>14.32</td>
<td>15.02</td>
</tr>
<tr>
<td>C</td>
<td>14.85</td>
<td>13.53</td>
<td>15.04</td>
</tr>
<tr>
<td>D</td>
<td>16.80</td>
<td>16.80</td>
<td>17.98</td>
</tr>
<tr>
<td>Total</td>
<td>13.82</td>
<td>13.32</td>
<td>14.52</td>
</tr>
</tbody>
</table>

**Table 2.** Isolation rates of *H. influenzae* according to medium, by laboratory

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geographical and demographic variation in incidence, as previous studies had shown similar tendencies and the laboratory with the lowest isolation rate for H. influenzae also had the highest isolation rate for S. pneumoniae. The quantity of H. influenzae growth was significantly greater on BCA. Of the 250 isolates where growth on both media could be compared, 47 yielded heavier growth on BCA, but only 6 yielded heavier growth on CBA (p < 0.0001).

Competing flora was significantly diminished on BCA (p < 0.0001) as shown in Table 3. Although a bacitracin disk on CBA was considered to be helpful in only 43% of cases where H. influenzae was present in heavy growth, it became a valuable tool in the recognition of light growths (Table 4).

Information on isolations of S. pneumoniae was available from only three laboratories. In the presence of S. pneumoniae, the isolation rate for H. influenzae was 19.7%, compared with 12.9% in the absence of S. pneumoniae (p = 0.09). Therefore, there was no evidence to indicate that the presence of S. pneumoniae had inhibited the growth of H. influenzae on CBA.

**Discussion**

In 1950, Finland and Wilcox [6] reported that the minimum inhibitory concentration (MIC) of bacitracin for 16 strains of H. influenzae ranged from 750 to 3000 mg/L. A further publication by Del Love and Finland [7] examined 90 strains, all of which were resistant to 1000 mg/L. Later, Barber [3] found that all tested strains of staphylococci, streptococci and neisseriae were inhibited by bacitracin 6400 U/L, whereas none of 26 Haemophilus isolates was inhibited by <25000 U/L. In the same study, ‘heated blood agar’ supplemented with bacitracin 10000 U/L increased isolation rates of H. influenzae from sputum by almost 30% compared with plain blood agar and an enriched, heated blood agar containing crystal violet. Hovig and Aandahl [4], in the same year, found that bacitracin concentrations of 300–3000 mg/L (18 900 U/L) or lower permitted full growth of all strains of Haemophilus spp. tested, and went on to compare CBA containing bacitracin 240 or 300 mg/L (15 100 or 18 900 U/L) with CBA and a bacitracin 10 U disk. The isolation rate of Haemophilus spp. rose from 14.6% on CBA with a bacitracin disk to 51.2% on the supplemented agar. However, Barber examined only 79 sputum samples and Hovig and Aandahl only 41 mixed sputum and throat swabs, and no attempt was made to differentiate H. influenzae from other Haemophilus spp.

Michaels and Stonebraker [8] reported that the concentration of bacitracin in their enriched Levinthal-based medium could be varied between 3000 and 20 000 U/L without reduction in either the growth of H. influenzae or the effectiveness of inhibition of competing flora. In an attempt to clarify the optimal concentration of bacitracin, Ederer and Schurr [9] compared the efficacy of 5000, 10 000 and 15 000 U/L, in an enriched BA. Consistently more profuse growth was detected on the medium with 5000 U/L and 15 000 U/L inhibited the growth of some strains. For this reason, bacitracin 5000 U/L is now the manufacturer’s recommended concentration for selection of H. influenzae and was used in this study. This concentration led neither to excessive breakthrough of competing flora nor inhibition of growth of H. influenzae. No data exist on the effect of varying concentrations of bacitracin in the disks. However, it is unlikely that concentrations below 10 U/disk would offer any advantage, given that, overall, the disk was helpful in only 42% of isolations. A higher disk content may risk inhibiting some strains of H. influenzae.

May [10] suggested that the true prevalence of H. influenzae in sputum samples might be masked by an abundance of S. pneumoniae; however, the findings of the present study do not support that view.

This large, multi-centre study shows that BCA and CBA with a bacitracin disk are equivalent for the isolation of H. influenzae. BCA plates are easier to read, with much fewer competing flora and more abundant growth. However, as BCA is a medium specifically for respiratory microbiology, it offers no economic advantage over CBA with a bacitracin disk.

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**Table 3.** Number of samples indicating presence or absence of competing flora by medium

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Present on BCA</th>
<th>Absent on BCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present on CBA</td>
<td>1165</td>
<td>441</td>
</tr>
<tr>
<td>Absent on CBA</td>
<td>39</td>
<td>345</td>
</tr>
</tbody>
</table>

**Table 4.** Assessment of helpfulness of a bacitracin disk for the isolation of H. influenzae

<table>
<thead>
<tr>
<th>Bacitracin disk</th>
<th>Growth (cfu/ml)</th>
<th>Helpful</th>
<th>Unhelpful</th>
<th>Not recorded</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^1–10^4</td>
<td>21</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>10^5–10^6</td>
<td>29</td>
<td>18</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>&gt;10^6</td>
<td>50</td>
<td>116</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>137</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

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**References**


