The novel use of tryptose sulfite cycloserine egg yolk agar for isolation of *Clostridium perfringens* during an outbreak of necrotising enterocolitis in a neonatal unit

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Running Title: Isolation of *C. perfringens* using TSC-EYA during a NEC outbreak
Clostridium perfringens has been associated with necrotising enterocolitis (NEC) which is a serious disease of neonates. Our study describes the novel use of selective tryptose sulfite cycloserine with egg yolk agar (TSC-EYA) during a nursery outbreak. This medium provides a rapid, sensitive and accurate presumptive identification of C. perfringens.
Necrotising enterocolitis (NEC) is the most common acquired disease affecting the gastrointestinal system of neonates, with low birth weight babies at highest risk (20, 22). Clinical features of NEC range from mild intestinal signs such as abdominal distension (Stage 1), radiological signs of pneumatosis (Stage 2), to advanced disease (Stage 3) involving severe abdominal distension, hypotension and peritonitis (1, 20). The underlying pathophysiology of NEC is poorly understood, but is likely to be secondary to multiple injuries to the neonate gut through hypoxia-ischemia, hyperosmolar feeds and infection (20, 24).

No single infectious agent has been consistently identified as a cause of NEC, but Enterobacteriaceae (Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae) viruses (rotavirus, coronavirus, echovirus, norovirus) and clostridial species have all been implicated (4, 23, 25, 30). The pathology of NEC resembles gas gangrene of the intestine caused by Clostridium perfringens, which produces a range of extracellular toxins (10, 11, 19, 27) and colonisation with C. perfringens has been shown to be associated with both sporadic and nursery outbreaks of NEC (2-4). However, it is unclear whether C. perfringens is the causative agent of NEC or a marker of intestinal changes associated with the disease (2, 3, 16).

Culture for C. perfringens is not usually undertaken for neonatal faeces as it is considered a part of the normal faecal flora with up to 35% of pre-term neonates colonised within the first two weeks of life (2, 30). In addition, isolation using conventional media such as horse blood agar (HBA) if required, is difficult without the use of selective supplements (2, 17). However, C. perfringens is also a major
cause of human food poisoning and when implicated in food-borne outbreaks the causative bacterium can be recovered and enumerated by the use of highly selective media such as tryptose sulfite cycloserine agar (TSC), which provides a rapid presumptive morphological identification of *C. perfringens* (6, 8, 9, 31).

The neonatal unit at Monash Medical Centre has capacity for 50 neonates and includes 18 level III (ventilated) neonatal intensive care unit (NICU) beds. The unit has a stable background NEC rate of about 6/1000 admissions per year. From 1st January to 30th June 2008, fifteen neonates were diagnosed with NEC (modified Bell Stage 2 and above) increasing the yearly rate to 32/1000 admissions and raising concerns of an outbreak. Cases were defined as neonates who met the NEC stage 2 or 3 criteria and controls were defined as current neonates who were in the unit without NEC at the peak June outbreak period (1).

Faecal samples were collected from 11 remaining neonates with NEC and 45 without NEC (current controls) from 6th to 20th June 2008. Microbiological investigations were undertaken for possible bacterial and viral pathogens (28).

Faecal samples (*n*=56) were also cultured for *C. perfringens* using HBA (Oxoid CM 0331) and incubated for 48 hours at 35°C in anaerobic jars. Colonies were examined for anaerobic haemolytic gram positive rods. Presumptive identification of *C. perfringens* was determined by using the reverse CAMP test (RC) (7, 12, 13). All isolates exhibiting haemolysis were confirmed using the RapidID Ana II panel (Remel, Kansas) and by 16S rRNA gene sequencing.
Faecal samples were also directly cultured onto TSC-EYA, which consisted of perfringens agar base (CM0587, Oxoid) with 5% egg yolk emulsion and D-cycloserine for 24 hours at 35°C (MPU, The University of Melbourne) (5, 9, 14, 15). Black, lecithinase positive or negative colonies were identified as presumptive *C. perfringens* and confirmed as already described. To aid in the recovery of clostridia from a background mixture of bacteria, faecal samples were also heat shocked at 60°C for 20 mins and then cultured onto TSC-EYA (17). Since some *C. perfringens* isolates are known to be heat sensitive a third method was used whereby samples were pre-treated with ethanol for one hour before being cultured onto TSC-EYA (17, 18, 21).

All phenotypic black colonies that exhibited lecithinase activity and were RC positive underwent 16S ribosomal RNA gene sequence analysis and Multiplex PCR toxinotyping. Preparation of genomic DNA from clostridial isolates and multiplex PCR including primer pair sequences used for genotyping was as previously described (26, 29).

Direct inoculation onto TSC-EYA was the most sensitive method examined, detecting an extra four culture positive neonates compared to standard culture on HBA (Table 1). Both heat shock and ethanol were less effective approaches for the isolation of *C. perfringens* compared to direct plating onto TSC-EYA. No positive stools were found from neonates negative on TSC-EYA using other methods. Direct plating on TSC-EYA was also rapid saving 24-48 hours compared to standard culture on HBA. In total, *C. perfringens* was isolated using TSC-EYA from 10 of 56 (18%) of study
subjects: 3 of 11 (27%) from NEC cases and 7 of 45 (16%) from controls (OR 1.69; p=0.46).

Multiplex PCR toxinoype analysis revealed that all *C. perfringens* isolates were type A, with the *plc* gene, which encodes α-toxin, being the only major typing toxin gene detected (data not shown).

Four other isolates that were reverse CAMP positive produced black colonies or were lecithinase positive on TSC-EYA were identified by Clostridial 16S rRNA gene amplification and sequence analysis (Table 2).

The rate of NEC in the unit returned to normal background levels over the second half of 2008 with only one NEC case diagnosed in December 2008. The outbreak resolved with implementation of enhanced infection control measures such as environmental cleaning, isolation and cohorting. An increased detection of norovirus was observed during this outbreak, but its etiological role in the pathogenesis of NEC has yet to be demonstrated (28).

In conclusion, our study numbers from this outbreak of NEC in a neonatal nursery are small and a larger multicentre study with prospective sampling needs to be conducted to establish if *C. perfringens* is a causative agent of NEC or a marker of intestinal changes preceding disease. However, direct inoculation of neonatal faecal samples onto TSC-EYA provides a sensitive, rapid and accurate presumptive identification of *C. perfringens* and would facilitate recovery in future investigations.
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Table 1. *Clostridium perfringens* isolated from neonatal faecal samples (n=56)

<table>
<thead>
<tr>
<th>Neonate</th>
<th>Sample Date</th>
<th>Standard Method HBA</th>
<th>Direct Method TSC-EYA</th>
<th>Heat Shock Method TSC-EYA</th>
<th>Ethanol Shock Method TSC-EYA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>11/06/08</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Case 2</td>
<td>19/06/08</td>
<td>ND</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Case 3</td>
<td>27/06/08</td>
<td>D</td>
<td>D</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Control 1</td>
<td>18/06/08</td>
<td>D</td>
<td>D</td>
<td>ND</td>
<td>D</td>
</tr>
<tr>
<td>Control 2</td>
<td>18/06/08</td>
<td>D</td>
<td>D</td>
<td>ND</td>
<td>D</td>
</tr>
<tr>
<td>Control 3</td>
<td>20/06/08</td>
<td>D</td>
<td>D</td>
<td>ND</td>
<td>D</td>
</tr>
<tr>
<td>Control 4</td>
<td>18/06/08</td>
<td>D</td>
<td>D</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Control 5</td>
<td>18/06/08</td>
<td>ND</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Control 6</td>
<td>18/06/08</td>
<td>ND</td>
<td>D</td>
<td>D</td>
<td>ND</td>
</tr>
<tr>
<td>Control 7</td>
<td>18/06/08</td>
<td>ND</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td><strong>Total recovered</strong></td>
<td></td>
<td>6</td>
<td>10</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>

TSC-EYA: Tryptose Sulfite Cycloserine Agar with egg yolk emulsion
D=Detected
ND=Not Detected
<table>
<thead>
<tr>
<th>Neonate</th>
<th>Sample Date</th>
<th>Standard Method</th>
<th>Direct Method</th>
<th>Heat Shock Method</th>
<th>Ethanol Shock Method</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>11/06/08</td>
<td>ND</td>
<td>B-, L+W</td>
<td>B-, L+&lt;sup&gt;W&lt;/sup&gt;, RC+&lt;sup&gt;W&lt;/sup&gt;</td>
<td>B-, L+, RC+&lt;sup&gt;W&lt;/sup&gt;</td>
<td>C.baratii</td>
</tr>
<tr>
<td>Control 1</td>
<td>18/06/08</td>
<td>RC+&lt;sup&gt;W&lt;/sup&gt;, BH-</td>
<td>B-, L+W</td>
<td>ND</td>
<td>B-, L+&lt;sup&gt;W&lt;/sup&gt;, RC+&lt;sup&gt;W&lt;/sup&gt;</td>
<td>C.sardiniense</td>
</tr>
<tr>
<td>Control 2</td>
<td>18/06/08</td>
<td>ND</td>
<td>ND</td>
<td>B+, L-, RC-</td>
<td>ND</td>
<td>C.paraputrificum</td>
</tr>
<tr>
<td>Control 3</td>
<td>20/06/08</td>
<td>ND</td>
<td>ND</td>
<td>B-, L+W, RC+&lt;sup&gt;W&lt;/sup&gt;</td>
<td>B-, L+, RC+&lt;sup&gt;W&lt;/sup&gt;</td>
<td>C.baratii</td>
</tr>
</tbody>
</table>

(TSC-EYA) Tryptose Sulfite Cycloserine with egg yolk
(HBA) Horse Blood Agar
(RC) Reverse CAMP test
(B) Black colonies (Hydrogen sulphide production)
(L) Lecithinase production on TSC
(BH) Beta haemolysis on Horse Blood Agar
<sup>W</sup> weak lecithinase on TSC
<sup>W</sup> weak haemolytic zone on Sheep Blood Agar
(+ ) positive
(- ) negative