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Novel Use of Tryptose Sulfite Cycloserine Egg Yolk Agar for Isolation of *Clostridium perfringens* during an Outbreak of Necrotizing Enterocolitis in a Neonatal Unit[▽]

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***Clostridium perfringens* has been associated with necrotizing 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*.**

Necrotizing 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), to 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 colonization 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 feces, as *C. perfringens* is considered a part of the normal fecal flora, with up to 35% of preterm neonates colonized within the first 2 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, which provides a rapid presumptive morphological identification of *C. perfringens* (6, 8, 9, 31).

The neonatal unit at Monash Medical Centre has the 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 6/1,000 admissions per year. From 1 January to 30 June 2008, 15 neonates were diagnosed with NEC (modified Bell stage 2 and above), increasing the yearly rate to 32/1,000 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).

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

Fecal samples ($n = 56$) were also cultured for *C. perfringens* using HBA (Oxoid CM 0331) and incubated for 48 h at 35°C in anaerobic jars. Colonies were examined for anaerobic hemolytic Gram-positive rods. Presumptive identification of *C. perfringens* was determined by using the reverse CAMP test (RC) (7, 12, 13). All isolates exhibiting hemolysis were confirmed using the RapID ANA II panel (Remel, Kansas) and by 16S rRNA gene sequencing.

Fecal samples were also directly cultured on tryptose sulfite cycloserine with egg yolk agar (TSC-EYA), which consisted of perfringens agar base (CM0587; Oxoid) with 5% egg yolk emulsion and D-cycloserine, for 24 h at 35°C (Media Preparation Unit, University of Melbourne) (5, 9, 14, 15). Black, lec-

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TABLE 1. *Clostridium perfringens* isolated from neonatal fecal samples

Neonate	Sample date (day/mo/yr)	No. of samples with isolation of *C. perfringens*[a]			
		Standard method on HBA	Direct method on TSC-EYA	Heat shock method on TSC-EYA	Ethanol shock method on TSC-EYA
Cases					
1	11/06/2008	D	D	D	D
2	19/06/2008	ND	D	D	D
3	27/06/2008	D	D	ND	ND
Controls					
1	18/06/2008	D	D	ND	D
2	18/06/2008	D	D	ND	D
3	20/06/2008	D	D	ND	D
4	18/06/2008	D	D	ND	ND
5	18/06/2008	ND	D	D	D
6	18/06/2008	ND	D	D	ND
7	18/06/2008	ND	D	D	D
Total recovered		6	10	5	7

[a] TSC-EYA, tryptose sulfite cycloserine with egg yolk agar; HBA, horse blood agar; D, detected; ND, not detected. Total number of samples, 56.

ithinase-positive or -negative colonies were presumptively identified as *C. perfringens* and confirmed as described above. To aid in the recovery of clostridia from a background mixture of bacteria, fecal samples were also heat shocked at 60°C for 20 min and then cultured on TSC-EYA (17). Since some *C. perfringens* isolates are known to be heat sensitive, a third method was used whereby samples were pretreated with ethanol for 1 h before being cultured on TSC-EYA (17, 18, 21).

All phenotypically black colonies that exhibited lecithinase activity and were RC positive underwent 16S rRNA gene sequence analysis and multiplex PCR toxinotyping. The preparation of genomic DNA from clostridial isolates and multiplex PCR including primer pair sequences used for genotyping were done as previously described (26, 29).

Direct inoculation onto TSC-EYA, which identified four more culture-positive neonates than did standard culture on HBA, was the most sensitive method examined (Table 1). Both heat shock and ethanol shock were less effective approaches for the isolation of *C. perfringens* than direct plating on TSC-EYA. No positive results were found by other methods using neonate samples that were negative on TSC-EYA. Direct plating on TSC-EYA was also rapid, saving 24 to 48 h compared to standard culture on HBA. In total, *C. perfringens* was isolated using TSC-EYA from 10 of 56 (18%) of the study subjects: 3 of 11 (27%) NEC cases and 7 of 45 (16%) controls (odds ratio [OR], 1.69; $P = 0.46$).

Multiplex PCR toxinotype analysis revealed that all *C. perfringens* isolates were type A, with the *plc* gene, which encodes alpha-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 (i.e., physically separating infected from uninfected neonates). 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 sample numbers from this outbreak of NEC in a neonatal nursery are small, and a larger, multicenter 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, di-

TABLE 2. Other *Clostridium* spp. recovered from neonatal fecal samples

Neonate	Sample date (day/mo/yr)	Isolate characteristics[a] identified by:				Species identification
		Standard method on HBA	Direct method on TSC-EYA	Heat shock method on TSC-EYA	Ethanol shock method on TSC-EYA	
Case 1	11/06/2008	ND	B^-, $L^{+(WL)}$	B^-, $L^{+(WL)}$, RC^{+WH}	B^-, L^+, $RC^{+(WH)}$	*C. baratii*
Control 1	18/06/2008	$RC^{+(WH)}$, BH^-	BH^-, $L^{+\ (WL)}$	ND	BH^-, $L^{+(WL)}$, $RC^{+(WH)}$	*C. sardiniense*
Control 2	18/06/2008	ND	ND	B+, L^-, RC^-	ND	*C. paraputrificum*
Control 3	20/06/2008	ND	ND	BH^-, $L^{+(WL)}$, $RC^{+(WH)}$	BH^-, L^+, $RC^{+(WH)}$	*C. baratii*

[a] TSC-EYA, tryptose sulfite cycloserine with egg yolk agar; HBA, horse blood agar; RC, reverse CAMP test; B, black colonies (hydrogen sulfide production); L, lecithinase production on TSC-EYA; BH, beta-hemolysis on HBA; WL, weak lecithinase production on TSC-EYA; WH, weak hemolytic zone on sheep blood agar; +, positive; −, negative; ND, not detected.

rect inoculation of neonatal fecal samples onto TSC-EYA provides a sensitive, rapid, and accurate presumptive identification of *C. perfringens* and will facilitate recovery in future investigations.

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There are no potential conflicts of interest.

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