Eur J Pediatr (2008) 167:891–895
DOI 10.1007/s00431-007-0614-9

ORIGINAL PAPER

Necrotizing enterocolitis of the neonate with Clostridium perfringens: diagnosis, clinical course, and role of alpha toxin

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Received: 11 June 2007 / Revised: 7 September 2007 / Accepted: 15 September 2007 / Published online: 20 October 2007

Abstract The severity of the clinical course in necrotizing enterocolitis (NEC) associated with *Clostridium perfringens* (Cp) may support the hypothesis of a specific disease. We conducted a case control study of infants diagnosed with NEC, who underwent surgical treatment over a 7-year period. Patient histories examined characteristics of the infants, bacterial infection as well as NEC's severity, antibiotic treatment, and clinical course. Infants infected with NEC associated with Cp were compared with NEC patients without Cp. The alpha toxin from Cp type A was detected in most of the isolated strains. Cp was identified as a causative agent of NEC in nine cases. As compared with the control group (n=32), the onset of disease was earlier in life, the clinical course more severe, and patients had a larger extent of gangrene. Portal venous gas was evident in 77% of all Cp cases, as compared to 25% in the control group. The mortality rate was 44% in the Cp group, and only 18.7% in the control group. Type A *Clostridium perfringens* was identified in six cases. In each isolate alpha toxin production was proven, but without any correlation to the severity of the clinical course, the extent of intestinal gangrene or mortality. In premature infants NEC in conjunction with Cp seems to be more severe than other NEC cases; it also entails higher mortality and morbidity. Alpha toxin concentrations do not correlate with the severity of the disease. Portal venous gas is highly suggestive for the diagnosis of Cp infection.

Keywords Necrotizing enterocolitis · Clostridium perfringens · Portal venous gas · Alpha toxin · Gas gangrene

Abbreviations

NEC	necrotizing enterocolitis
Cp	*Clostridium perfringens*
DIC	disseminated intravascular coagulation

Introduction

Necrotizing enterocolitis (NEC) is one of the leading causes of mortality in preterm infants. The disease is characterised by an acute bowel inflammation of different extents and localisations, with or without perforation. The pathogenesis of the disease is not yet clearly defined, but has so far been characterised by a multifactorial origin with imbalance of the bacterial gut flora with overgrowth of pathogenic bacteria and ischaemia causing mucosal lesions giving

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pathogenic bacteria systemic access [9, 10, 12, 19]. Reports describing the isolation of *Clostridium perfringens* (Cp) from infants with NEC suggest that these microorganisms may play an important role in the pathophysiology of the disease [18]. Ischaemia of the bowel might trigger the conversion of clostridial spores into toxin-producing invading bacilli. Tissues with reduced vascular supply and therefore low oxygen tension favour a rapid bacterial multiplication with subsequent exotoxin release. The alpha type is the most common cause of human gas gangrene [8].

This report represents a review of nine newborns suffering from a fulminant form of NEC associated with a Cp infection in which the alpha toxin is produced. The specific course and outcome of these NEC cases with higher morbidity and mortality rates as compared with known NEC cases without Cp might define a new variety of NEC. This may lead to new preventive and therapeutic approaches.

Patients and methods

Case histories of all infants with a NEC diagnosis who were admitted to our institution from January 1999 to December 2006 for surgical intervention were eligible for this retrospective study. Patients' records were reviewed in order to characterise the clinical findings, disease progression, and outcome. The data gathered from each infant were: birth weight, gestational age, onset of symptoms, feeding prior to onset of NEC, radiographic (Bell classification [2]) and/or ultrasonic findings, the extent of gangrene, and outcome. The clinical course was described as shock followed by disseminated intravascular coagulation (DIC), and finally haemolysis.

Patients in whom Cp was proven were compared with patients without Cp in terms of these clinical data.

Laparatomy was indicated by pneumatosis intestinii in conjunction with clinical deterioration, portal venous gas, and radiological signs of perforation. Segments of intestine with significant gangrene, which were removed during surgery, were examined macroscopically and histologically.

Bacteriologic cultures from blood, stool, and the operation site were obtained from each case either at the first sign of NEC symptoms or during surgical intervention. The samples were plated on Columbia Blood Agar with aztreonam discs to suppress the gram-negative aerobes, Schaedler Blood Agar, and Thiogycolad Broth (Haifa, Israel, Heidelberg, Germany). Haemolytic gram-positive rods were determined with the RapID Ana II panel (Remel, Kansas).

Clostridium perfringens isolates from six patients were genotypically subtyped with multiplex PCR according to Meer and Songer [14]. This PCR allows a specific detection of the toxongenes *cpa, cpb, etx, iap,* and *cpe*. The specific beta2-toxingene detection was performed by a Monoplex-PCR. Measurement of bacterial alpha toxin production was done by ELISA technique (Bio-X ELISA, Jenelle, Belgium) according to the manufacturer's instructions.

Statistics Patients with positive Cp results were randomised into one group; all others were randomised in a control group. Mean values, maxima, and minima were calculated for gestational age and birth weight. Both groups were compared with Fisher's exact test and t-test. Significant difference was assumed if $p<0.05$. Odds ratio was calculated for the items "intestinal perforation", "portal venous gas", and "mortality".

The protocol of the study was subject to ethical review by the local institutional review board.

Results

From 1999 to 2006, a total of 41 patients diagnosed with NEC were admitted to our unit for surgery. All infants underwent laparotomy. Cp could be isolated in bacteriologic cultures taken from nine neonates. They were randomised into the Cp group. Eight patients had positive cultures for Cp from the peritoneal cavity, and in two cases the organism was isolated from blood cultures. In only one case was the germ additionally found in the stool. Seven of the nine patients were transferred from other hospitals; two patients came from our unit. There was no evidence of epidemic outbreaks because the cases were registered sporadically over time. In 6 cases of 32 infants from the control group, no infectious agent associated with NEC could be found. In ten patients, a combination of bacteria and viruses was isolated from stool, operation site, or blood culture, and 16 infants displayed evidence of infection with gram-negative or gram-positive bacteria other than Cp.

There was no significant difference in gestational age in either group. However, the patients of the Cp group had a higher birth weight than those of the control group (Table 1). The onset of NEC was also different between the two groups. In the Cp infants symptoms started earlier on day 2 of life through day 13 of life (Table 1).

The clinical presentations of NEC in both groups were quite similar: abdominal distention, microscopically to grossly bloody stools, gastric residuals, and pneumatosis intestinalis. However, infants with Cp infection had intestinal perforations and portal venous gas (seven out of nine cases) more often. Portal venous gas was evident in only 25% of control patients.

Five infants of the Cp group did not receive any antibiotic agents prior to NEC, whereas four patients of

Table 1 Characteristics of patients with NEC and detectable Cp compared with patients suffering from NEC, but without Cp infection

	Cp group n=9	Control group n=32	p	Odds ratio (95% CI)
Mean gestational age (weeks, range)	30.1 (24–37)	28.9 (22–37)	0.41	
Mean birth weight (g, range)	1614 (640–3070)	1295 (530–2355)	0.17	
Onset of NEC (days of life, range)	9.8 (2–38)	22.4 (4–59)	0.08	
Intestinal perforation n (%)	6 (66%)	8 (25%)	0.04	6.0 (1.3–27.11)
Portal venous gas	7 (78%)	8 (25%)	0.006	10.5 (2.10–52.35)
Extent of gangrene				
Mild (n)	0	13	0.03	
Moderate (n)	1	8	0.65	
Severe (n)	8	11	0.006	
Mortality n (%)	4 (44%)	6 (18, 7%)	0.18	3.46 (0.73–16.42)

Severity of the gangrene depends upon the length of resection, where "mild" signifies that no bowel resection is needed; "moderate" signifies a small resection; "severe" signifies a large resection.

this group were treated with different antibiotic regimens. Twenty-eight patients in the control group received different antibiotics within the first 3 days of life; four infants never received any antibiotic treatment prior to the onset of NEC. Two patients of the Cp group were fed mother's milk before onset of the symptoms, six were fed formula milk, and one infant had exclusively parenteral nutrition prior to NEC. In the control group, 26 patients were fed orally prior to NEC with different types of formula and 6 received parenteral nutrition.

All patients in the Cp group were classified into Bell stage II and higher. Rapid clinical deterioration required surgical intervention within the first 24 h after the onset of symptoms in all cases. Gangrene of the bowel was seen in each case to different extents: in the Cp group gangrene was severe in eight of nine cases, and moderate in one. In contrast, most of the infants of the control group had milder forms of gangrene (Table 1). According to the extent of tissue damage, resection of the necrosis was performed. In six cases of the Cp group, an additional perforation of the gut was recorded. Histopathologic results confirmed a diagnosis of NEC.

Eight of the nine patients with Cp NEC had fulminant shock after surgery, in four cases combined with DIC, acute renal failure in two, and haemolysis in four cases. Four patients survived and four patients died within the first 96 h after surgical intervention. The mortality rate of the control group was lower: 6 out of 32 (18.7%) infants died in the context of NEC.

Cp isolate subtyping from the last six patients of our series revealed the presence of type A strains, which produce the alpha toxin. The alpha toxin could be quantified in all of these infants. There seemed to be no correlation between toxin concentration and outcome; the infant with the lowest toxin concentration had a lethal outcome and the infant with the highest toxin concentration survived (Table 2).

Discussion

The etiology of NEC is not well defined. Besides prematurity and impaired intestinal blood circulation, which are known risk factors, bacterial infection with subsequent inflammation plays a major role [16]. A large variety of bacteria, viruses, and fungi associated with NEC suggest that there is no specific infectious promoter of the disease [17]. However, there is evidence from animal models that some *Clostridia* strains–including Cp–are responsible for specific NEC-like lesions [25].

Several studies describing *Clostridium* isolation in NEC cases also report the severity of the clinical course associated with this organism. The purpose of our study was to support the hypothesis that Cp infection is not simply coincidental to NEC, but rather defines a specific disease. This thesis agrees with the findings of Koloske et al. [11], demonstrating the relationship between Cp and a specific fulminant clinical course of NEC. Their patients also had an onset early in life (within the first 5 days) as well as a higher mortality in their Cp group (78%). The severity of disease documented during surgical intervention was also 100%.

Table 2 *Clostridium perfringens* type A, detection of the toxin production in vitro and the severity of the clinical course in five cases. (Index [1] optic density of the toxin concentration in relation to bacterial concentration)

Patient no.	Source of the isolated CP cultures	Alpha toxin index [1]	Extent of gangrene	Outcome
4	Stool	0.92	Severe	Exitus letalis
5	Operation site	1.29	Severe	Exitus letalis
6	Operation site	2.63	Severe	Exitus letalis
7	Operation site	1.2	Severe	Survival
8	Operation site	4.2	Moderate	Survival
9	Operation site	1.17	Severe	Survival

The clinical course of our study was characterised by an early onset in the first 2 weeks of life with a rapid progression after the onset of the first symptoms, and by pneumatosis intestinalis, portal venous gas, or radiological signs of perforation. Portal venous gas was useful for diagnosing NEC caused by Cp.

The clinical presentation of NEC is different in extremely premature infants as compared with more mature infants [21]. However, the clinical signs of NEC with Cp were the same across the age groups. Portal venous gas, a radiographic sign which indicates a serious clinical course with poor prognosis [15], was more often seen in babies with Cp-related NEC. The previously mentioned short interval between the onset of symptoms and surgery was attributed to the short incubation time of the organism [27]. Intraoperatively, extensive gangrene necessitated bowel resection in each case. This was significantly different to findings in non-Cp-NEC cases. Postoperatively, systemic complications were documented in eight out of nine patients, and consisted of shock, renal failure, haemolysis, and coagulopathy. Mortality associated with Cp-NEC with Cp was more than double as high as in infants suffering from NEC with either other specific agents or no bacteria and viruses.

The role of bacterial toxins in the pathogenesis of NEC is still under discussion [20]. Whereas the *Clostridium difficile* toxin can be detected in the feces of healthy newborns [23], the virulence of Cp type A strains correlates with toxin production, in which α-toxin plays the starring role in terms of gas gangrene and varities of enterocolitis in humans as well as in several animals [13, 24]. Therefore we measured the production of α-toxin in these type A strains, which were isolated from six patients. Three samples were not available at the point of investigation. To our knowledge, this is the first time that a quantification of the alpha toxin production in Cp has been performed. Alpha toxin plays a key role in the pathogenesis of *C. perfringens* type A strain-related diseases such a gas gangrene in humans [8] and in animals [13]. Alpha toxin shows phospholipase C activity and haemolytic activity as well as a variety of subtle effects in the intracellular metabolism of eukaryotic target cells, which confirm its toxicity [4, 7, 26].

Alpha toxin production could be proven in all of the six isolates tested, but there was no correlation between the amount of alpha-toxin production in vitro and clinical course and outcome of the cases. The isolate with the highest alpha toxin production originated from a patient with a moderately severe clinical course only, lacking the systemic effects of myocardial suppression and haemolysis. In contrast, the strain with the lowest toxin concentration in vitro had been isolated from a patient who developed shock symptoms and had a lethal outcome.

The inappropriate colonisation of the premature intestine had been cited as causing NEC [3]. Bacterial colonisation of the neonatal gut begins by contact with the vaginal flora and is stimulated by oral feedings and exposure to the postpartum environment [5]. One report from Finland [1] describes how the intestinal flora is colonised with Cp in the presence of gastrointestinal disorders like flatulence, distended abdomen, diarrhea, etc., but without NEC in neonates. Still, early Cp colonisation seems to be correlated with NEC in premature infants [6], where intrapartum antibiotics and gut flora imbalance favour the growth of Cp. As compared with our patients suffering from Cp, only four NEC patients received intra- or post-partum antibiotics.

The mucosal barrier of premature infants is insufficient during the first weeks of life, and its higher permeability might not only be due to local bacterial toxin effects [26]. In our study, patients with Cp saw the onset of symptoms during the first 2 weeks of life. In veterinary medicine, bowel diseases caused by Cp type A are well known [22]; in newborn animals, the onset of disease is most likely to be within the first 2 weeks of life, with a fulminant course and fatal or near-fatal outcome similar to the human disease.

Vaccination against Cp alpha toxin, which can be used in animals [24], may play no role in the high risk group of premature infants because of their specific immunological situation and the sporadic exacerbation, as well as for practical reasons. Other preventive approaches include cautious hygenic handling, which is not specific to Cp, but all other infections with bacteria or other pathological agents.

With a fulminat course of NEC especially with portal venous gas on a X-ray film one should always consider the possibility of a Cp infection. In this case the addition of penicillin G to the therapeutic regime should be considered before culture results are available.

In conclusion, NEC caused by Cp infection is a very severe form of enterocolitis of the preterm infant with high mortality and a high risk of intestinal damage. Portal venous gas is a sensitive clinical sign in the diagnosis of Cp-linked NEC. In this case, an expanded antibiotic therapy, including penicillin G, may be useful. Further investigation will need to clarify the amount of the biologically active toxin in vivo in correlation with the outcome of the disease.

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