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Necrotoxigenic *Escherichia coli* type-2 invade and cause diarrhoea during experimental infection in colostrum-restricted newborn calves

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Abstract

There exists experimental evidence that necrotoxigenic *Escherichia coli* (NTEC) strains producing the cytotoxic necrotising factor 1 cause intestinal and extra-intestinal disease in piglets. On the other hand, no experimental model has been developed with NTEC strains producing the cytotoxic necrotising factor 2. In all, 14 colostrum-restricted calves were orally challenged with two strains isolated from the faeces of a diarrheic calf (B20a) or from the heart blood of a septicaemic calf (1404). All calves had diarrhoea which lasted until euthanasia in eight of them. In those calves, diarrhoea was correlated with the faecal excretion of the challenge strains. At necropsy, vascular congestion of the intestinal mucosa, hypertrophy of the mesenteric lymph nodes (MLN) and some congestion of the lungs were observed. Bacteriology confirmed the colonisation of the intestine by the challenge strains which were also recovered from the heart blood, the lungs and/or the liver. Histological sections confirmed enterocolitis, lymphadenitis and limited bronchopneumonia. In the intestinal tissue sections, bacteria testing positive in an in situ DNA hybridisation assay with a CNF2 probe were observed. Those results were confirmed by immunohistochemistry with a polyclonal anti-O78 and a monoclonal anti-F17b antisera. Three of the five control calves receiving either saline or a CNF^{-}, F17a strain (25KH09) had no clinical signs or lesions. The other two presented a profuse liquid diarrhoea but those calves were positive for the presence of $K99^{+}$ *E. coli*. In this model, both NTEC2 strains were thus, able to colonise the intestine, to cause long-lasting diarrhoea and to invade the blood stream with localisation in various internal organs in colostrum-restricted conventional newborn calves.

Keywords: *Escherichia coli*; Cattle-bacteria; Cytotoxic necrotising factor

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1. Introduction

In 1983, Caprioli and collaborators described a cytotoxin produced by an *Escherichia coli* strain isolated from infant enteritis, causing multinucleation and enlargement in HeLa cells cultures and necrosis in rabbit skin. For these reasons, this factor was named cytotoxic necrotising factor or CNF (Caprioli et al., 1983) and CNF-producing *E. coli* were later called necrotoxigenic *E. coli* (NTEC) (Gonzales and Blanco, 1989). Two different types of CNF have actually been reported (De Rycke et al., 1987, 1990a): type-1 is similar to the one described by Caprioli et al. (1983), with formation of rounded multinucleated cells, whereas type-2, identical to the Vir toxin (Smith, 1974) causes the formation of elongated multinucleated cells. However, CNF1 and CNF2 are closely related antigenically and genetically (De Rycke et al., 1987; Oswald et al., 1994a). The *cnf*2 gene is located on the Vir plasmid (Smith, 1974; Oswald et al., 1994a), whereas the *cnf*1 gene is located on the chromosome (Falbo et al., 1992).

There is in vitro and in vivo evidence that NTEC strains are pathogenic and that CNF toxins are putative virulence factors. In vitro, data originate from studies on cell cultures. Although purified CNF toxins cause the formation of multinucleated cells, the effect of the interaction between NTEC2 strains and HeLa cells is characterised by the production of giant mononucleated cells with the appearance of actin stress fibres and a block in the G2/M phase of the division (De Rycke et al., 1996). The actin cytoskeleton reorganisation with the appearance of stress fibres is mediated by the CNF2 toxin, which causes hyperactivation of a small GTP binding Rho protein (Oswald et al., 1994a). The cell cycle block in the G2/M phase is caused by a more recently described toxin, the cytolethal distending toxin type-3 (CDTIII toxin), which prevents the activation of the cdc2 protein kinase (Comayras et al., 1997; Pérès et al., 1997).

In vivo evidence originates from field and experimental studies. The association of NTEC strains and disease in animals was recently reviewed (De Rycke et al., 1999). NTEC1 strains have been isolated from cases of enteritis in ruminants, pigs, dogs, rabbits and horses, from septicaemia in pigs, from urinary tract infections, and from other extra-intestinal infections in dogs, cats and humans. De Rycke and Plassiart (1990b) investigated the effect in young lambs of intravenous injection of a partially purified preparation of CNF1. All the lambs developed severe clinical signs starting 6 h after inoculation, which consisted mainly in neurological signs and mucoid diarrhoea. Orally-inoculated NTEC1 strains also cause enterocolitis and bacterial colonisation of the lungs in piglets (Wray et al., 1993; Clement et al., results not published).

NTEC2 strains have been isolated mainly from cattle, lambs (Blanco et al., 1996a) and goats (Cid et al., 1996) with intestinal infections but also with septicaemia, pneumonia, metritis, mastitis and abortion (Pohl et al., 1997) and only exceptionally from non-ruminants, such as rabbits, mares and cats with intestinal and extra-intestinal infections (Pohl et al., 1993; Blanco et al., 1996b). Since the experiments of Smith (1975) with an ovine Vir *E. coli* strain, no experimental evidence has been reported on the involvement of NTEC2 strains or the role of CNF2 in diseases in calves. In addition, many NTEC2 strains have been isolated from healthy animals (Pohl et al., 1997; Blanco et al., 1998) showing the need for the development of a controlled model in calves.

This manuscript describes the clinical signs and lesions observed in newborn colostrum-restricted calves inoculated orally with two different NTEC2 strains.

2. Materials and methods

2.1. Bacterial strains

NTEC2 strain B20a was isolated from the faeces of a calf with diarrhoea (Oswald et al., 1991). This strain is serotype O15:K14, is serum resistant, produces an aerobactin (Oswald et al., 1991) and harbours genes encoding F17c and d fimbriae (Mainil et al., 2000). NTEC2 strain 1404 was isolated from the blood of a calf with septicaemia (Oswald et al., 1991). This strain is serotype O78:K80, produces an aerobactin (Oswald et al., 1991) and harbours genes encoding for the F17b fimbriae (Mainil et al., 2000). Prior to the challenge, the pathotypes of these two strains were checked by colony hybridisation with the appropriate gene probes (Mainil et al., 1997) and their serotypes by agglutination with specific antisera. Strain 25KH09 originally isolated from the faeces of a calf with diarrhoea (Pohl et al., 1982), is serotype O101:K^+:H^- (Pohl et al., 1987), and is the reference strain for the F17a fimbriae (Lintermans et al., 1988a). This strain is considered non-pathogenic and was chosen as an adherent non-toxigenic control (Pohl et al., 1987; Pohl and Mainil, 1995).

2.2. Experimental protocol

In all, 19 Holstein Friesian calves born in conventional conditions were removed from their dam immediately after birth. They were kept one after the other in a clean stall disinfected each time with Atlantol (ECO.SA, Ghent, Belgium). They received 300 ml of colostrum during the first hour of life and were then fed with commercial UHT-sterilised full cream milk twice a day. The colostrum was tested by agglutination and by ELISA for the absence of activity to strains B20a, 1404 and 25KH09.

Six agar slants were plated with the respective challenge strain. After a 6 h growth, the inoculum was obtained by resuspension and pooling of the bacteria in 250 ml of sterile saline (Kaeckenbeek, personal communication) in order to obtain an optical density (620 nm) of 0.08 corresponding to a challenge dose of 10^9 colony forming units (cfu's) ml^{-1}. Serial dilutions of the inoculum were plated onto Gassner-agar plates (Merck, Darmstadt, Germany) with a spiral plater (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK) and incubated overnight at 37°C to verify the number of bacteria inoculated. The totality of the suspension (250 ml) was orally administrated to the calves using a sterile bottle with a teat.

The first three calves were inoculated at 24 h of age with strain 1404. Subsequently, the calves were inoculated at 6 h of age: four calves with strain 1404, seven with strain B20a, and one with strain 25KH09. Four control calves received 250 ml of sterile saline without bacteria and were kept one after the other in the same conditions (housing and feeding) as the inoculated ones. The animals were followed clinically and faeces were collected every 4 h from inoculation time until euthanasia. Oral secretions, nasal secretions and urine were collected twice daily for bacterial examination.

Table 1
Correlation between the presence of the diarrhoea and the challenge strain in the faeces[a]

Calves	Challenge strain	Inoculation time	Euthanasia (hPi)	Diarrhoea		Challenge strain in the faeces	
				Appearance (hPi)	Persistence until	Appearance	Persistence until
1	1404	24	64	11	E	20 hPi	36 hPi
2	1404	24	60	18	26 hPi	18 hPi	30 hPi
3	1404	24	73	14	24 hPi	16 hPi	24 hPi
12	1404	06	51	28	36 hPi	24 hPi	28 hPi
13	1404	06	50	28	E	36 hPi	E
14	1404	06	48	28	E	24 hPi	E
27	1404	06	46	40	E	28 hPi	E
5	B20a	06	65	36	48 hPi	36 hPi	48 hPi
6	B20a	06	86	24	46 hPi	24 hPi	28 hPi
7	B20a	06	36	28	E	24 hPi	E
8	B20a	06	74	28	E	28 hPi	E
9	B20a	06	39	28	E	24 hPi	E
10	B20a	06	45	40	E	28 hPi	E
11	B20a	06	57	32	E	32 hPi	E
c1	–	NR	–	–	–	NR	NR
c2	–	NR	45	–	–	NR	NR
c3	25KH09	06	60	–	–	12 hPi	E
c4	–	NR	48	24	E	NR[b]	NR[b]
c5	–	NR	52	20	E	NR[b]	NR[b]

[a] Hours post-inoculation: hPi; euthanasia: E; not relevant: NR; none or negative: –.
[b] Excretion of K99$^+$ *E. coli*.

2.3. Necropsy

The calves were killed at various post-inoculation (Pi) times (Table 1) by parenteral injection of sodiumpentobarbital 20% and necropsied immediately. The thoracic cavity was first opened and macroscopic lesions were recorded. Samples of heart blood and lungs were removed for bacteriological and/or histopathological examination. Secondly, the abdominal cavity was opened, the macroscopic lesions recorded and samples of organs (liver, spleen, kidneys, intestine and mesenteric lymph nodes (MLN)) were removed for bacteriological and histopathological examination. Finally, 200 μl of intestinal content (duodenum, jejunum, ileum, caecum, colon) were collected for bacterial examination. The whole necropsy was performed within 1 h.

Tissues were fixed in a phosphate-buffered formaldehyde/glutaraldehyde solution (4/1, v/v), processed routinely, paraffin-embedded and sections of 5 μm were obtained. Tissue sections were then stained with hematoxylin–eosin (HE) and examined for presence of bacteria and inflammatory lesions.

For immunohistochemistry, tissue sections were stained by Vector red (Vector Laboratories, Burlington, Ont., Canada) as described by Roger and co-workers (1996) with a rabbit polyclonal anti-O78 antiserum (P. Pohl, Veterinary and Agrochemical

Research Centre, Brussels, Belgium) and a mouse monoclonal anti-F17b antiserum (H. Ball, Department of Veterinary Science of the Queen's University, Belfast, Northern Ireland, UK).

2.4. Bacteriology

The presence of coliforms in faeces and intestinal contents was quantitatively estimated after inoculation of Gassner-agar plates and overnight incubation at 37°C of serial dilutions as described for the inoculum. In addition, the proportion of the challenge strains among lactose-fermenting colonies was estimated quantitatively by colony hybridisation of a plate with radioactive probes for CNF2 (Oswald et al., 1994b), F17 (Lintermans et al., 1988b) or K99 (Mainil et al., 1986) as described below. The presence of coliforms in extra-intestinal tissues, nasal secretions, oral secretions, urine and heart blood was estimated qualitatively: a tissue sample or a drop was directly plated onto Gassner-agar and Colombia with 5% sheep blood-agar plates (Becton-Dickinson, Meylan, France). The plates were incubated overnight at 37°C and colonies were identified by colony hybridisation. Diarrheic faecal samples were also examined for the presence of coronavirus, rotavirus, *Cryptosporidium parvum* and $K99^+$ *E. coli* using a Bio-X Trousse ELISA Digestive (Bio-X, Brussels, Belgium) and for the presence of salmonellae as follows: one drop was diluted in tetrathionate broth (Oxoid, Basingstoke, England), incubated 48 h at 42°C and subsequently plated onto Gassner-agar plates, lactose non-fermenting colonies were agglutinated with a OMA test (Sanofi, Marnes-la Coquette, France) and positive colonies were confirmed with API 20 E sugarsets (bioMerieux SA, Marcy l'Etoile, France).

2.5. DNA colony hybridisation

Whatman 541 paper filters (Whatman International Ltd., Maidstone, England) were placed onto colonies after overnight growth on Gassner-agar plates as explained before. After 2 h, the paper filters were peeled off and treated with the following solutions: 10% sodium dodecyl-sulphate (SDS) for 3 min; 0.5 M NaOH/1.5 M NaCl (pH 12.8) for 15 min and 1.0 M Tris/1.5 M NaCl (pH 7.6) for 2×5 min, allowed to dry and hybridised overnight at 65°C with the DNA probes for CNF2, F17 or K99 (Mainil et al., 1997). After washing, filters were autoradiographed overnight. The Gassner-agar plates with the inoculum were treated at the same time and used as indirect positive controls.

2.6. In situ hybridisation

The CNF2 probe was produced by PCR as follows. DNA was released from strain 1404 by boiling. Bacteria (300 μl) were harvested from an overnight Luria–Bertani (LB) broth culture and centrifuged for 30 s. The pellet was suspended in 50 μl of sterile water, incubated at 100°C for 10 min and centrifuged for 30 s. The supernatant was amplified by Taq DNA polymerase (Boerhinger, Mannheim, Germany) with primers B121: (CAA GAA CAA TGA ACG GGT AA) and B122 (AAA AAT GCT GGG ATA GTA AC) (GIBCO BRL, Paisley, UK). The 143 bp amplicon was purified (PCR purification kit, Qiagen, Hilden, Germany) and digoxin-labelled (DIG Nick translation kit, Boerhinger).

Unstained paraffin-embedded tissue sections of intestine, lungs, liver, spleen and MLN were pre-treated as described by Lewis and Wells (1992). The probe was diluted in hybridisation buffer (2X SSC, 5% (w/v) dextran sulphate, 0.2% (w/v) milk powder, 50% formamide) at a concentration of 200 ng ml^{-1} and was denatured at 100°C for 10 min while the slides were heated at 95°C for 10 min using a heating block. The probe (50 μl) was applied to the centre of the slides. The slides were incubated overnight at 37°C in a humidified box and washed as follows: 2X SSC at 60°C for 20 min with agitation, 0.2X SSC at 42°C for 20 min with agitation, 0.1X SSC at room temperature for 5 min, 2X SSC at room temperature (Lewis and Wells, 1992). The slides were immersed in 0.3% H_2O_2 for 45 min, washed in 50 mM Tris–HCl pH 7.4 (TBS), incubated in TBS containing 10% horse serum and 1% bovine serum albumin (BSA) for 30 min. The liquid was discarded and the anti-DIG antibody (Boerhinger) was added overnight at 4°C. The slides were washed in TBS. The detection was performed using the diaminobenzidine (DAB) detection kit (Boehringer). The slides were mounted using Permount (Fisher Chemical, Fair Lawn, NJ, USA).

2.7. Pulse-field gel electrophoresis (PFGE)

E. coli strains were grown overnight in 5 ml of LB at 37°C. The optical density of the culture was subsequently adjusted to 0.8 at 620 nm with EET buffer (Na_2EDTA 100 mM, EGTA 10 mM, Tris–HCl 10 mM pH 8). This suspension (50 μl) was centrifuged at 12 000 × *g* for 5 min at 4°C. The supernatant was removed and the pellet was suspended in 50 μl of EET buffer. This suspension was mixed with 50 μl of 2% low-melting-temperature agarose (Gibco BRL, Paisley, UK) previously put at 80°C and then pipetted into a plug mold (BioRad Laboratories, Richmond, CA, USA) and allowed to solidify. For lysis, the plugs were placed in 500 μl of deproteinisation solution (EET buffer, pronase 20 mg ml^{-1}, SDS 20%). Following overnight incubation at 37°C, the plugs were washed six times for 30 min with TE (Tris–HCl 10 mM, EDTA 1 mM) pH 8 and then stored at 4°C. Digestion was performed with enzyme XbaI. A portion of the plugs (1/4) was placed in a microcentrifuge tube with 100 μl of reaction buffer for 30 min Then, the buffer was removed, replaced by 50 μl of reaction buffer with 2 μl of enzyme and the tubes were incubated at 37°C. After 5 h, the supernatant was removed and replaced by 250 μl of EDTA 0.5 M. The plugs were then loaded into the wells of a 1% pulsed-field certified agarose gel (BioRad) in 0.5X TBE buffer (BioRad). The gel was processed by using the CHEF MAPPER apparatus (BioRad). An electrophoretic regimen of 6 V cm^{-1} for 21 h at 14°C and switch times of 2–20 s was used. The gel was then stained with ethidium bromide for 45 min, destained 30 min in water and photographed with UV-illumination.

3. Results

3.1. Clinical signs

The inoculation at 24 h of age was followed by the appearance of some diarrhoea between 11 and 18 hPi. The diarrhoea persisted until euthanasia of the first calf but disappeared after 8 h for the other two (Table 1).

Eight of the eleven calves inoculated with the NTEC2 strains (1404, B20a) at 6 h of age presented watery diarrhoea, which appeared between 24 and 40 hPi and persisted until euthanasia (Table 1). Five calves (C7, C8, C9, C10 and C14) showed apathy, anorexia and dehydration, two (C8 and C14) respiratory distress and one (C7) was dying at the time of euthanasia. Short-lasting diarrhoea was observed in the other three calves (C5, C6 and C12). Two calves (C6 and C12) also had generalised clinical signs including apathy, anorexia and tachycardia, became recumbent and were dying when euthanised.

No diarrhoea was observed in three control calves uninoculated (c1, c2) or inoculated with strain 25KH09 (c3). They appeared clinically healthy and behaved normally (Table 1). On the other hand, a profuse liquid diarrhoea was observed in the other two control calves (c4, c5) which persisted until euthanasia.

3.2. Excretion of the challenge strains

The NTEC2 challenge strain 1404 was recovered from faecal cultures of the three calves inoculated at 24 h of age for a maximum period of time of 16 h (Table 1).

The NTEC2 challenge strains (1404, B20a) were recovered from faecal cultures of the 11 calves inoculated at 6 h of age (Table 1). In all but three calves (C10, C13 and C27), the faecal excretion of the NTEC2 strain coincided with or was observed 4 h before the appearance of diarrhoea (Table 1). In all but three calves (C5, C6 and C12), the faecal excretion persisted until euthanasia. Strain 25KH09 was recovered from the faeces from 12 hPi until euthanasia (Table 1). The numbers of the NTEC2 strains present in the faeces as well as their proportion among *E. coli* were highly variable and were not correlated to the severity of clinical signs (Tables 2 and 3). No bacteria were recovered from nasal and oral secretions nor from urine (data not shown).

All inoculated calves were negative for the presence of cryptosporidiae, $K99^+$ *E. coli* and salmonellae; only calf C12 was positive for the presence of rotavirus and coronavirus (data not shown). On the other hand, the two non-inoculated calves with diarrhoea (c4 and c5) were positive for the presence of $K99^+$ *E. coli*.

Table 2
Faecal excretion of the 1404 strain in log cfu's ml^{-1} of faeces[a]

Time (hPi)	C1	C2	C3	C12	C13	C14	C27
0–16	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
16	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20	**8.1**	**8.0**	**8.5**	N.D.	N.D.	N.D.	N.D.
24	**8.5**	**7.2**	**7.9**	10.9	N.D.	7.3	N.D.
28	**8.5**	**7.2**	**7.7**	**10.9**	N.D.	**9.6**	8.5
32	**8.6**	N.D.	N.D.	N.D.	N.D.	**10**	8.9
36	**8.6**	N.D.	N.D.	N.D.	**10.9**	**10.1**	9.0
40	N.D.	N.D.	N.D.	N.D.	**11.1**	**10.1**	**9.3**
44	N.D.	N.D.	N.D.	N.D.	**11**	**10.2**	**9.3**

[a] The calf was dead at the period 46–73 hPi; the challenge strain was not detected: N.D.; values in bold type show presence of diarrhoea.

Table 3
Faecal excretion of the B20a strain in log cfu's ml^{-1} of faeces[a]

Time (hPi)	C5	C6	C7	C8	C9	C10	C11
0–24	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
24	N.D.	**8.9**	7	N.D.	10.4	N.D.	N.D.
28	N.D.	**8.6**	**9.1**	**8.5**	**10.9**	9.4	N.D.
32	N.D.	N.D.	**8.7**	**10.4**	**11**	9.7	**9.7**
36	**8.9**	N.D.	[b]	**10.8**	**10.5**	9.1	**10.2**
40	**8.9**	N.D.		**10.8**	[b]	**8.6**	**9.8**
44	**8.6**	N.D.		**11.8**		**8.6**	**9.6**
48	**8.2**	N.D.		**11.4**		[b]	**9.2**
56	N.D.	N.D.		**10.5**			[b]
60	N.D.	N.D.		**10.2**			
64	N.D.	N.D.		**9.9**			
68	[b]	N.D.		**9.3**			
72–86		[b]		[b]			

[a] The challenge strain was not detected: N.D.; values in bold type show presence of diarrhoea.
[b] Calf was dead at that time.

3.3. Necropsy and microscopic lesions

At necropsy, all calves inoculated with the NTEC2 strains had lesions of vascular congestion and thickening of the intestinal mucosa in all parts of the small intestine (which were more pronounced in the jejunum and ileum), and hypertrophy of the associated MLN. Petechial haemorrhages were present in the hearts of C7 and C12 and C12 also had lesions of fibrinous arthritis in the hocks.

No macroscopic lesions were observed in two out of five control calves (c2 and c3). In control calves c4 and c5, lesions of vascular congestion of the intestinal mucosa were present but localised in the jejunum and in the ileum only. The first control calf (c1) was not euthanised.

The small intestine of all the NTEC2 inoculated calves and two control calves (c4 and c5) showed congestion (Fig. 1A) and atrophy of the villi. High infiltration of inflammatory cells (neutrophils, lymphocytes and plasmocytes) was present in the mucosa (Fig. 1A) of all these calves and in the submucosa of C6, C8, c4 and c5. It also showed necrosis and/or abscessation of the crypts and lymphatic canals were distended. Peyer's patches showed activation in jejunum and ileum. Bacteria were observed associated with the mucosa of the intestinal villi or the intestinal content (Fig. 1D).

The caecum and the colon of these calves also showed congestion and infiltration of inflammatory cells (lymphocytes and neutrophils) in the mucosa of all calves and in the submucosa and tunica muscularis of calves c4 and c5. The crypts were necrosed in the colon of the calves C6, C11, C12, c4 and c5. The colon of the control calves c4 and c5 showed activation of the Peyer's patches.

In these calves, the MLN showed activation of lymphoid nodules and contained neutrophils from the cortical sinus to the medulla. The lungs showed neutrophils in the interstitium or in the bronchiolar and alveolar lumens accompanied by congestion and

interalveolar oedema (Fig. 1C). The spleen also showed activation of the lymphocytic population, interlaced with neutrophils in some calves (C9, C11, C12, C13, C14, C27, c4 and c5). Lymphocytes and neutrophils were also observed in the liver of calves C10, C13, C14 and C27.

No abnormalities other than congestion were found in the internal organs of one of the uninoculated control calves (c2) (Fig. 1B). The 25KH09 inoculated calf (c3) showed slight inflammation with atrophy of the villi, lymphocyte infiltration of the small intestine and activation of lymphoid nodules. No abnormalities other than congestion were found in the other organs of this calf.

3.4. Bacteriology at necropsy

The NTEC2 challenge strains were recovered in high numbers (10^6 to 10^9 cfu's ml^{-1} of intestinal content) from the various parts of the intestines of the eight calves challenged at 6 h of age in which diarrhoea persisted till the time of euthanasia (Table 4). The NTEC2 challenge strains were also recovered in pure culture from the heart blood, the lungs and/or the liver of the same calves, but not from the kidneys, the spleen or the MLN (Table 5). On the other hand, the NTEC2 challenge strains were not recovered from the intestine of calves C1, C2, C3, C5, C6 and C12 (Table 4), neither from their internal organs (Table 5). However, CNF2 probe-negative *E. coli* strains were isolated from the heart blood of three of them (calves C5, C6 and C12) and from several internal organs of calves C6 (spleen, liver, kidneys and nodes) and C12 (lung, liver, kidneys and joint synovia). Those strains were confirmed as different from the challenge strains by using pulse-field gel electrophoresis (data not shown).

E. coli 25KH09 was also recovered in high numbers from the various parts of the intestine of calf c3 (Table 4) but not from its internal organs (Table 5). In the same way, a $K99^+$ *E. coli* was also recovered in high numbers from the intestine of calves c4 and c5 (Table 4) but not from their internal organs (Table 5).

3.5. In situ hybridisation

DNA hybridisation with the CNF2 probe derived by PCR was performed on the tissue sections of the intestine and the lungs of calf C7 inoculated with NTEC2 strain B20a and calves C13 and C14 inoculated with NTEC2 strain 1404. The same tissue sections of calf c3 inoculated with CNF-negative strain 25KH09 were used as negative controls. Positive hybridisation results were observed in the intestine of calves C7 and C14. Bacteria were associated with the intestinal content or with the epithelial cells of the intestinal villi (Fig. 2). No positive results were observed in any internal organs.

3.6. Immunohistochemistry

Immunohistochemistry with a rabbit polyclonal anti-O78 antiserum was performed on the tissue sections of the intestine, the lungs and the liver of calves C13 and C14 inoculated with NTEC2 strain 1404. Positive results were observed in the intestine of calf C14. Positive bacteria were associated with the intestinal content or with epithelial cells

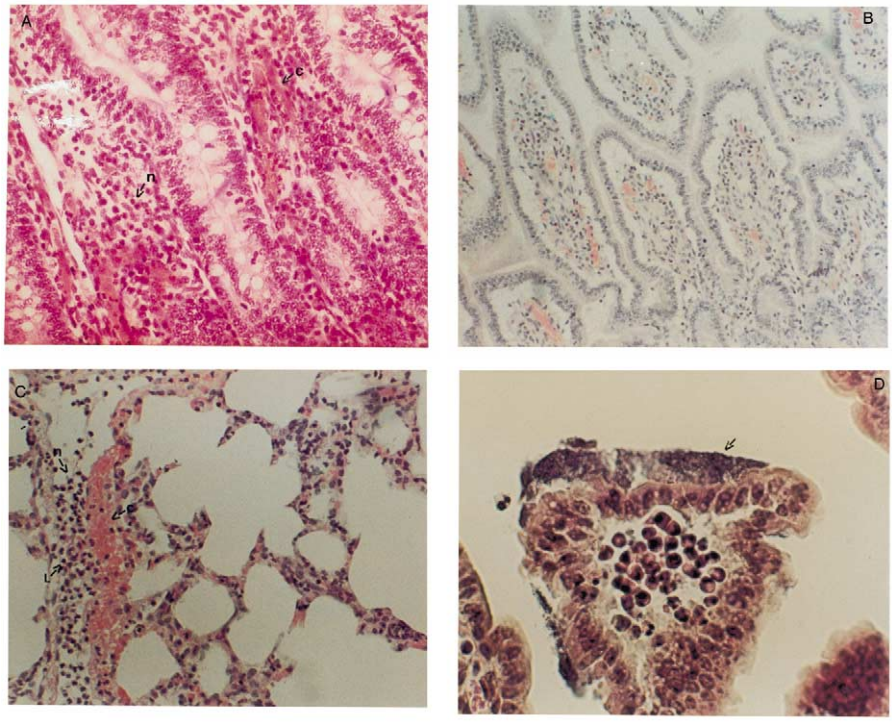

Fig. 1. (A) Ileum of C13 (HE 128×): enteritis, presence of congestion (c) and neutrophils (n). (B) Ileum of the control calf C2: no abnormalities but congestion were detected. (C) Lung of C13 (HE 128×): pneumonia, presence of congestion (c), neutrophils (n) and lymphocytes (l). (D) Jejunum of the C7 (HE 240×): bacteria associated with the intestinal villi (arrow).

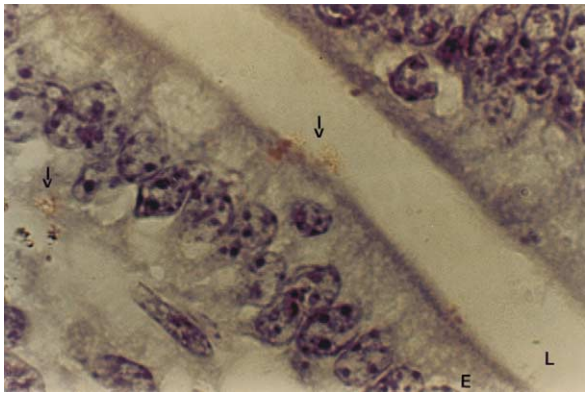

Fig. 2. Detection of $CNF2^{+}$ bacteria on ileum sections by in situ hybridisation (staining, DAB; counterstaining, hematoxylin, 240×): positive bacteria are localised at the top and inside the enterocytes (arrow); L, intestinal lumen; E, enterocyte.

Table 4
Recovery of the challenge strain from the intestinal content at necropsy[a] in log cfu's ml^{-1} of faeces[b]

Calves	Duodenum	Jejunum	Ileum	Caecum	Colon
1	–	–	–	–	–
2	–	–	–	–	–
3	–	–	–	–	–
12	–	–	–	–	–
13	6.8	7.9	8.2	8.1	7.7
14	7	8	8.1	8.6	8.1
27	7.2	9.3	9.5	8.6	8.7
5	–	–	–	–	–
6	–	–	–	–	–
7	6.7	7.4	8.4	7.4	8
8	6.6	7.8	8.4	8.4	7.6
9	6.5	8.5	8.1	8.9	7.9
10	6.8	8.2	8.5	8.4	8.4
11	6	8	8.6	8.2	8
c3	10.5	10.7	10.6	10.7	10.5
c4	9.3[c]	9.9[c]	10.8[c]	10.1[c]	9.8[c]
c5	9.5[c]	10.3[c]	10.5[c]	10.1[c]	9.9[c]

[a] The time of the euthanasia is mentioned in Table 1.
[b] Absence of NTEC2 strain in the whole culture: −.
[c] Recovery of the contaminating $K99^+$ *E. coli* in log cfu's.

Table 5
Recovery of the challenge strain from the extra-intestinal organs at necropsy[a]

Calves	Blood	Lung	Liver
1	−	−	−
2	−	−	−
3	−	−	−
12	−	−	−
13	+	+	+
14	+	+	+
27	−	−	+
5	−	−	−
6	−	−	−
7	+	+	−
8	+	+	−
9	−	+	−
10	+	+	+
11	+	−	−
c3	−	−	−
c4[b]	−	−	−
c5[b]	−	−	−

[a] Presence of NTEC2 strains: +; absence of NTEC2 strains: −.
[b] Recovery of the contaminating $K99^+$ *E. coli*.

of the intestinal villi. Those results were confirmed using a mouse monoclonal anti-F17b antiserum. No positive results were observed in any internal organs.

4. Discussion

The first step in the understanding of the role of the CNF2 and CDTIII toxins in the diseases associated with NTEC2 in ruminants is the setting-up of an experimental model of disease reproduction in calves as it was done in NTEC1 in piglets (Clement et al., results not published).

In eight calves out of eleven inoculated at 6 h of age (calves C7, C8, C9, C10, C11, C13, C14 and C27), diarrhoea persisted until the time of euthanasia and was correlated with the excretion of high numbers of the challenge strain in the faeces (Tables 1–3) and with its presence in the intestinal content at necropsy (Table 4). However, no correlation was observed between the severity of the diarrhoea and the degree of excretion of the challenge strain. As no other classical diarrhoeagenic agents were isolated, since three control calves had no clinical signs at all, and as the diarrhoea in two other control calves was caused by a $K99^+$ *E. coli*, it can be assumed that NTEC2 strains were the actual cause of the diarrhoea observed in B20a and 1404 inoculated calves after they successfully colonise the gut of those calves.

These calves also presented signs of generalised bacterial infection (bacteraemia or septicaemia), and the challenge NTEC2 strains were isolated from the heart blood and from internal organs (lungs and/or liver), but not from the MLN confirming the specific invasive character of the NTEC2 strains. The invasive character seem to be a specific property of the NTEC2 strains, as testified by the absence of strains 25KH09 and of contaminating $K99^+$ *E. coli* in internal organs of calves C3 to C5, although not an exclusive one since non-NTEC *E. coli* were also isolated from internal organs of three calves (C5, C6, and C12). These observations are in general agreement with observations in piglets inoculated with NTEC1 strains, showing the predilection for the lung associated in some cases with diarrhoea, respiratory signs and death (Wray et al., 1993; Fournout et al., 2000; Clement et al., results not published).

Histological lesions were present in the intestinal wall and in internal organs. The lesions of the intestine are characteristics of an enteritis with abscessation and necrosis of the crypts. The presence of abscesses and neutrophil clusters in the intestine suggests a bacterial cause. This observation is also supported by the presence of neutrophils in nodes and spleen and by the pulmonary lesions. The lesions were more severe in the small intestine than in the large intestine. Surprisingly, few bacteria were observed by light microscopy and those that were detected were associated with the intestinal epithelium or with the mucus mainly. Similar observations were made in piglets infected with NTEC1 strains (Fournout et al., 2000; Clement et al., results not published). The lesions in internal organs consisted mainly in neutrophilic infiltration.

The diarrhoea observed and the excretion of the challenge strain did not last long in three additional calves inoculated at 6 h of age with strain B20a (C5 and C6) or 1404 (C12) nor in three calves inoculated at 24 h of age with strain 1404 (C1, C2 and C3). In addition, the challenge strain was not recovered at necropsy either from the intestinal

content or from extra-intestinal sites. The reasons for the failure to colonise the intestines of these calves is unknown, perhaps (i) the genetic background of the animals with the absence of expression of receptor, or (ii) the absence or poor expression of the colonisation factor, in this case, F17 fimbrial adhesins, or (iii) the age of the animals with disappearance of enterocytes's receptors like for K99 adhesin (Runnels et al., 1980). Moreover, although all possible precautions were taken to avoid infections with other pathogen organisms, rotavirus and coronavirus were detected in the faeces of calf C12, non-NTEC2 *E. coli* which are different from the challenge strain by PFGE from the heart blood and/or from the internal organs of calves C5, C6 and C12. Failure of colonisation by the NTEC2 strain may thus, also be the consequence of the presence of another *E. coli* strain in the intestine prior or concomitantly to the challenge.

The mechanism of passage of the NTEC2 strains through the intestinal wall is unknown, but it is tempting to speculate on a role for the CNF2 toxin, since NTEC2 are seen in close association with enterocytes and since the effect of the toxin on cultivated epithelial cells is a reorganisation of the F-actin cytoskeleton (Oswald et al., 1994a). Actin cytoskeleton rearrangement is indeed a mean for a non-invasive bacteria to be internalised into epithelial cells (Falzano et al., 1993).

According to the results obtained so far, NTEC2 strains are able to colonise the intestine and to cause persistent diarrhoea in ''colostrum-restricted'' conventional newborn calves. Moreover, those strains are able to invade the blood stream, most probably after crossing the intestinal wall, and to localise in the internal organs.

However, it must be emphasised that restriction of colostrum, age at the time of inoculation and absence of other invasive *E. coli* appear to enhance the ability of the NTEC2 to colonise the gut and invade the blood stream. NTEC2 may thus, represent opportunistic pathogens waiting for favourable circumstances (like newborn), a consideration which in no way diminishes their virulence potential once the circumstances are present. Similar observations were made recently in piglets with NTEC1 (Clement et al., results not published).

This model will be used in the future to test NTEC mutants in toxin- and/or adhesin-encoding genes to investigate more specifically their role in the pathogenesis of diarrhoea and/or invasion, as it was recently done with NTEC1 strains in piglets (Fournout et al., 2000). Precise interaction between NTEC2 and mutants strains and the intestinal epithelium may also be investigated in more details using intestinal explants (Baehler and Moxley, 2000).

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