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# Characteristics of necrotoxigenic *Escherichia coli* isolated from septicemic and diarrheic calves between 1958 and 1970

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**Abstract**

A total of 434 *Escherichia coli* isolated from septicemic calves between 1958 and 1965 and 430 *E. coli* isolated from diarrheic calves between 1967 and 1970 were studied by colony hybridisation and PCR assays for the presence of the *cnf1*- and the *cnf2*-like genes. They were also studied for the presence of genes coding for putative virulence factors associated with the CNF toxins including F17-, Pap- and Sfa-fimbrial adhesins and the recently described CDT-III toxin and AfaVIII-afimbrial adhesin. Thirty (7%) of the 434 septicemic strains were positive for CNF by colony hybridisation. Twenty-six were confirmed as necrotoxigenic *E. coli* type 2 (NTEC2) and four as NTEC1 by PCR. Thirty-five (8%) of the 430 diarrheic strains were positive for CNF by colony hybridisation. Five of them were studied by PCR and confirmed as NTEC1. The 26 septicemic NTEC2 strains and 20 of the 35 diarrheic NTEC including three of the five NTEC1 were positive for CDT-III. All adhesins studied were present in NTEC as well as in non-NTEC. NTEC1 were mainly Pap-, Sfa- and/or Afa8-positive, whereas NTEC2 were mainly F17- and/or Afa8-positive. This study shows that necrotoxigenic *E. coli* with their associated adhesins and toxins were present in calves as early as 1958, but their prevalence seems to have increased since that time. 

*Keywords:* *Escherichia coli*; Necrotoxigenicity; Cattle–bacteria

## 1. Introduction

Necrotoxigenic *Escherichia coli* are an emerging category of pathogenic bacteria. They are defined on the basis of production of a toxin called cytotoxic necrotising factor (CNF)

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(Caprioli et al., 1983). This toxin was named CNF for its capacity to induce cell multinucleation in Vero and HeLa tissue culture and to cause necrosis in rabbit skin (De Rycke et al., 1990). Two different types of NTEC have been reported: NTEC1 and NTEC2 (De Rycke et al., 1987, 1990). NTEC1 strains have been isolated from cases of enteritis in ruminants, pigs, dogs, rabbits and horses and from extra-intestinal infections in pigs, dogs, cats and humans (De Rycke et al., 1999). NTEC2 strains have been mainly isolated from ruminants with intestinal infections or with septicemia (Blanco et al., 1996; Cid et al., 1996; Pohl et al., 1997). The necrotoxigenic *E. coli* produce two different toxins (CNF1 and CNF2) closely related antigenically and genetically (De Rycke et al., 1987; Oswald et al., 1994a).

In 1989, Oswald and collaborators identified CNF2 as the Vir toxin of *E. coli* harbouring the Vir plasmid (Smith, 1974). The *cnf2* gene is indeed carried by the Vir plasmid (Oswald et al., 1994a), whereas the *cnf1* gene is located on the chromosome (Falbo et al., 1992).

Genes located on the Vir plasmid encode other putative virulence factors. The family of the F17 fimbriae, produced by *E. coli* isolated from animals and human beings with septicemia and/or diarrhea (Le Bouguenec and Bertin, 1999), include several antigenic subtypes: F17a, F17b, F17c and F17d. The F17b fimbrial subtype was identified as the Vir adhesin which is coded by genes located on the Vir plasmid as can the F17c fimbrial subtype (Oswald et al., 1991; El Mazouari et al., 1994; Mainil et al., 1997, 2000). A new member of the afimbrial adhesin AfaVIII, was recently identified in *E. coli* from cattle (Mainil et al., 1997; Lalioui et al., 1999). The *afa-8* gene cluster was demonstrated to be also located on the Vir plasmid in NTEC2 and chromosome-located in other *E. coli* including NTEC1 (Mainil et al., 1997; Gérardin et al., 2000). Finally, a member of the cytolethal distending toxins, CDT-III (Pérès et al., 1997) produced by NTEC2 is coded by genes located on the Vir plasmid.

On the other hand, most of the NTEC1 isolates harbour sequences typical of the P-fimbrial adhesin family and either one S-fimbrial (Sfa or F1C) (Fournout et al., 2000) or the AfaVIII-afimbrial adhesins (Mainil et al., 1997, 1999; Lalioui et al., 1999; Gérardin et al., 2000), which are all located on the chromosome. The *pap*/*prs* gene clusters coding for P-fimbriae and the *cnf1* gene are actually associated with a *hly* operon coding for the α-hemolysin, forming the so-called pathogenicity island 5 or Pai 5 (Blum et al., 1995; De Rycke et al., 1999; Dozois and Curtiss III, 1999).

The purpose of the present work was to determine the prevalence of NTEC1 and NTEC2 among *E. coli* isolated from diseased calves before their description by Smith (1974) as well as their other putative virulence factors. This was performed by hybridising collections of invasive and intestinal *E. coli* from the 1950s and 1960s with gene probes for the different adhesins and toxins described here above.

## 2. Materials and methods

### 2.1. E. coli strains

All *E. coli* strains used in this study originated from the collections of the Laboratory of Bacteriology, Faculty of Veterinary Medicine, University of Liège.

A total of 434 strains were isolated from heart blood or internal organs of septicemic calves. They were collected in Belgium between 1958 and 1965 (Kaeckenbeeck and Thomas, 1960) and preserved in a freeze–dried form.

A total of 430 strains were isolated from faeces of diarrheic calves. They were collected in Belgium between 1967 and 1970 (Schoenaers and Kaeckenbeeck, 1973) and preserved in agar deeps. In 1987, they were transferred onto Whatman 541 paper filters (Whatman International Ltd., Maidstone, England) (Mainil et al., 1990).

Five *E. coli* strains were used as positive or negative controls: S5 (CNF2+, CDTIII+, F17+); A30 (AfaIII+); J96 (CNF1+, Pap+, Sfa+); 239KH89 (CNF1+, AfaVIII+) and HS (negative control).

### 2.2. Serogroups

The serogroups of the septicemic strains were determined at the time of isolation using antisera to somatic antigens O (O1–O142) (Kaeckenbeeck and Thomas, 1960).

### 2.3. Gene probes

Six gene probes were used. The CNF probe was derived by a Pst1 + Cla1 restriction of the pEOSWO1 plasmid. The 335 bp fragment hybridises with both CNF1- and CNF2-producing strains (Oswald et al., 1994b). The other five probes were derived by PCR as described in the Section 2.5. The Sfa probe was derived from the strain 536, it corresponds to the *sfaD* and *sfaE* genes (Le Bouguenec et al., 1992). The Pap probe was derived from the strain C1212 and consists of a fragment internal to the *papC* gene (Le Bouguenec et al., 1992). The F17 probe was derived from the strain 25KH09 and corresponds to the major subunit *f17a* (Lintermans et al., 1988; Bertin et al., 1996). Those three probes are family probes. The Afa probe was derived from the strain 239KH89 and consists of an internal fragment of the *afaE* gene of the *afa-8* gene cluster (Lalioui et al., 1999). This probe is specific for AfaVIII. The CDT probe, which was derived from the *cdtIII* genes of the NTEC2 strain 1404 (Pérès et al., 1997) with specific primers (GAA AAT AAA TGG AAT ATA AAT GTC CG and TTT GTG TCG GTG CAG CAG GGA AAA), cross-hybridises with the *cdtII* genes (Oswald, personal communication).

### 2.4. DNA colony hybridisations

Septicemic *E. coli* were grown overnight at 37°C in Luria–Bertani broth (neutralised peptone 10 g/l; yeast extract 5 g/l; NaCl 10 g/l; pH 7.5) and subsequently overnight on Gassner-agar medium (Merck, Darmstad, Germany). They were transferred onto Whatman paper filters, which were prepared as previously described (Mainil et al., 1990). The filters with fecal *E. coli* were prepared in 1987 (Mainil et al., 1990).

All those filters were hybridised overnight at 65°C with each probe (Mainil et al., 1990). After washing, they were autoradiographed for 1–7 days.

### 2.5. PCR assays for cnf1 and cnf2

Bacteria were incubated in Luria–Bertani broth at 37°C overnight. 300 μl were centrifuged at 13,000 rpm for 30 s. The pellet was suspended in 50 μl of sterile water, incubated at 100°C for 10 min and centrifuged. The supernatant was used in the PCR reaction. Each microtube (Eppendorf, Hamburg, Germany) contained 1 IU of Taq DNA Polymerase; 5 μl of PCR Nucleotide Mix 10 mM; 5 μl of Taq DNA Polymerase Buffer 10X; 0.5 μl of each primer (40 nM) and 5 μl of DNA. The reaction mixture was overlaid with mineral oil (Nujol Mineral Oil, Perkin-Elmer, Branchlung, NJ, USA). The PCR was performed with a thermal cycler (Perkin-Elmer Cetus, Branchlung, NJ, USA) at 94°C for 10 min followed by 25 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The amplified product was visualised by gel electrophoresis using 10 μl of the final reaction mixture on a 2% agarose (Life Science Internationals, Zelik, Belgium) gel in TAE buffer (40 mM Tris, 20 mM acetic acid, and 2 mM EDTA pH 8.3). The samples were electrophorised for 60 min at 70 V. Amplified DNA fragments were located by UV fluorescence after staining with ethidium bromide.

A 0.75 kb band is amplified with both CNF2- and CNF1-producing strains (primers TTA TAT AGT CGT CAA GAT GGA and CAC TAA GCT TTA CAA TAT TGA) and a 1.25 kb band is amplified only with CNF2-producing strains (primers TAT CAT ACG GCA GGA GGA AGC ACC and GTC ACA ATA GAC AAT AAT TTT CCG). Therefore, a positive response with both pairs of primers is associated with CNF2-producing strains whereas a positive response only with the 0.75 kb band is associated with CNF1-producing strains (Oswald, personal communication).

## 3. Results

### 3.1. Toxin probes

Out of 434 septicemic strains, 30 (7%) hybridised with the CNF probe. Twenty-six strains were identified as NTEC2 by the PCR assay and four were identified as NTEC1. The 26 NTEC2 strains hybridised with the CDT-III probe. All the remaining strains (NTEC1 or non-NTEC) were negative with this probe (Table 1).

Table 1
Colony hybridisation results among the septicemic strains

| *E. coli* | Total number of strains | Number of positive strains with the probes | | | | |
|---|---|---|---|---|---|---|
| | | CDT | F17 | AfaVIII | Pap | Sfa |
| NTEC2 | 26 | 26 (100%) | 18 (69%) | 18 (69%) | 2 (7%) | 5 (19%) |
| NTEC1 | 4 | 0 | 1 (25%) | 3 (75%) | 4 (100%) | 4 (100%) |
| Non-NTEC | 404 | 0 | 225 (56%) | 211 (51%) | 105 (26%) | 15 (4%) |
| Total | 434 | 26 | 244 | 232 | 111 | 24 |

Table 2
Colony hybridisation results among the diarrheic strains

| *E. coli* | Total number of strains | Number of positive strains with the probes | | | | |
|---|---|---|---|---|---|---|
| | | CDT | F17 | AfaVIII | Pap | Sfa |
| NTEC (1 and 2) | 35 | 20 (57%) | 11 (31%) | 22 (62%) | 15 (43%) | 3 (8%) |
| NTEC1[a] | 5 | 2 (40%) | 3 (60%) | 3 (60%) | 3 (60%) | 0 |
| Non-NTEC | 395 | 0 | 72 (18%) | 86 (21%) | 62 (16%) | 5 (1%) |
| Total | 430 | 20 | 83 | 108 | 77 | 8 |

[a] Only five strains could be recovered and PCR identified as NTEC1.

Out of 430 diarrheic strains, 35 (8%) hybridised with the CNF probe. Unfortunately, only five of them were recovered from the agar deeps. They were identified by PCR as NTEC1. Twenty strains hybridised with the CDT-III probe (Table 2). All of them were CNF-positive strains and two of them were among the five strains confirmed as NTEC1 by PCR.

### 3.2. Serogroups

Fourteen serogroups were recognised among the 30 septicemic NTEC strains (Table 3). The most common among the 26 NTEC2 strains were O78 (23%) and O15 (15%). The serogroups of three of the four NTEC1 were O18, O23 and O117. Five NTEC2 and one NTEC1 were not typeable with the antisera used (Kaeckenbeeck and Thomas, 1960).

Table 3
O serogroups of septicemic strains

| O serogroup[a] | NTEC1[b] | NTEC2[b] |
|---|---|---|
| O2 | – | 1 |
| O15 | – | 4 |
| O18 | 1 | – |
| O23 | 1 | – |
| O45 | – | 1 |
| O55 | – | 1 |
| O63 | – | 2 |
| O78 | – | 6 |
| O86 | – | 1 |
| O88 | – | 1 |
| O103 | – | 1 |
| O117 | 1 | 1 |
| O132 | – | 1 |
| O140 | – | 1 |
| NT[c] | 1 | 5 |

[a] From Kaeckenbeeck and Thomas (1960).
[b] Number of strains with the serogroup.
[c] Not typeable with the antisera used.

### 3.3. Hybridisation of NTEC strains with adhesin probes

#### 3.3.1. Septicemic strains

All four NTEC1 and 25 NTEC2 tested positive with at least one of these probes (Table 1). All NTEC1 were Pap+ Sfa+ and three were AfaVIII+ also. The 25 NTEC2 were F17+ (seven strains), AfaVIII+ (seven strains) or F17+ AfaVIII+ (11 strains); six of them being also Sfa+ and/or Pap+.

#### 3.3.2. Diarrheic strains

Only 29 out of the 35 fecal NTEC tested positive with at least one adhesin probe (Table 2). Gene hybridisation profiles were typical of NTEC2: F17+ and/or AfaVIII+ (14 strains) or of NTEC1 (including the five strains PCR identified as NTEC1): Pap+ and Sfa+ or AfaVIII+ (10 strains). A few atypical results were observed: F17+ Pap+ (two strains); F17+ AfaVIII+ Pap+ (two strains); F17+ AfaVIII+ Pap+ Sfa+ (one strain) (Table 4).

### 3.4. Hybridisation of non-NTEC strains with adhesin probes

Many non-NTEC strains also tested positive with the adhesin probes (Tables 1 and 2).

Of the septicemic strains, a majority was positive with the F17 probe (56%) followed by the AfaVIII probe (51%) and the Pap probe (26%). Only a minority hybridised with the Sfa probe (4%) (Table 1). The most prevalent pathotypes were

Table 4
Pathotypes of the isolates

| Pathotypes | Septicemic strains[a] | | Diarrheic strains[a] | |
|---|---|---|---|---|
| | NTEC[b] | Non-NTEC | NTEC[c] | Non-NTEC |
| F17 | 7 | 27 | 3 | 55 |
| AfaVIII | 6 | 31 | 7 | 32 |
| Pap | – | 19 | – | 22 |
| Sfa | – | 5 | – | – |
| F17/AfaVIII | 6 | 117 | 4 | 19 |
| F17/Pap | – | 32 | 2 | 6 |
| F17/Sfa | – | – | – | – |
| AfaVIII/Pap | – | 4 | 7 | 14 |
| AfaVIII/Sfa | 1 | 9 | 1 | 1 |
| Pap/Sfa | 1 | – | 2 | – |
| F17/AfaVIII/Pap | 1 | 49 | 2 | 8 |
| F17/AfaVIII/Sfa | 3 | – | – | – |
| AfaVIII/Pap/Sfa | 2 | 1 | – | 1 |
| F17/AfaVIII/Pap/Sfa | 2 | – | 1 | – |
| Total number | 30 | 404 | 35 | 395 |

[a] Number of strains with the pathotype.
[b] 4 NTEC1 and 26 NTEC2.
[c] Only five of them could be PCR identified as NTEC1.

F17+ AfaVIII+ and F17+ AfaVIII+ Pap+. The others were present in <10% of the strains (Table 4).

The same probes were hybridised by the diarrheic strains, but in lesser proportion: AfaVIII (21%), F17 (18%), Pap (16%), and Sfa (1%). The most prevalent pathotype was F17+. The other pathotypes were present in <10% of the strains (Table 4).

## 4. Discussion

The cytotoxic necrotising factors (CNF) 1 and 2 were described, respectively in 1983 (Caprioli et al., 1983) and in 1974 (Smith, 1974; Oswald et al., 1989). Actually, Necrotoxigenic *E. coli* (NTEC) are considered as emerging pathogens (De Rycke et al., 1999). Using genetic tools, we were however, able to show that 7–8% of invasive and fecal *E. coli* isolated from diseased calves between 1958 and 1970 are NTEC strains. Most of the invasive NTEC are NTEC2 (26 strains of 30), but only five out of the 35 fecal NTEC were recovered and identified as NTEC1.

Nevertheless, the proportions of NTEC strains reported in more recent studies (Burns et al., 1996; Pohl et al., 1997; reviewed in De Rycke et al., 1999) are much higher. These differences can be explained by (a) the type of the animal populations sampled as for instance the risk of NTEC infection increases after the first month of life (Orden et al., 1999); or (b) the changes throughout the years in animal husbandry as intensive rearing, which can favour the dissemination of bacterial pathogens, has increased since the early 1960s; or (c) the decrease of incidence of other pathogens because of husbandry and vaccinal prophylaxis as for enterotoxigenic *E. coli*; or (d) the selection of resistant clones and/or plasmids by extensive antibiotic use; or (e) an increased awareness and the apparition of better laboratory techniques.

Only the serogroups of invasive *E. coli* were determined. The O serogroups recognised vary, but the most frequently observed were O78 and O15; which are indeed frequently associated with bovine NTEC2 strains (Burns et al., 1996; Mainil et al., 1999). On the other hand, serogroups observed among NTEC1 are not common (De Rycke et al., 1999; Mainil et al., 1999).

Not only did the *cnf1* and *cnf2* genes exist before 1983 and 1974, but DNA sequences related to genes coding for the other putative virulence factors of NTEC were also already present: *f17* described in ovine and bovine *E. coli* by Smith (1974) as Vir adhesin and re-described later under the name F17b (El Mazouari et al., 1994); *pap*/*prs* and *sfa*/*foc* described in human uropathogenic *E. coli* in 1980 and 1986, respectively (Johnson, 1991) and for the first time in bovine *E. coli* in early 1990s (Blanco et al., 1990; Harel et al., 1991); *cdt-III* described in 1997 (Pérès et al., 1997); and most recently *afa-8* (Lalioui et al., 1999).

These DNA sequences, were thus, present in invasive and fecal NTEC and non-NTEC, but were much more frequent among the septicemic strains, emphasising their potential role in extra-intestinal rather than intestinal pathologies. Most NTEC1 and many NTEC2 actually harbour several other properties of invasive *E. coli* (De Rycke et al., 1999; Mainil et al., 1999).

NTEC strains and their putative virulence factors, thus, existed prior to their published description. The difference observed resides in their frequency.

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