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Necrotoxigenic *Escherichia coli* from sheep and goats produce a new type of cytotoxic necrotizing factor (CNF3) associated with the *eae* and *ehxA* genes

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Summary. Fecal samples from sheep and goats were screened by tissue-culture assays and PCR for the presence of necrotoxigenic *Escherichia coli* (NTEC) producing cytotoxic necrotizing factors (CNFs). Of the 18 NTEC strains assayed, four were positive for the *cnf1* gene while 14 strains were negative for the *cnf1* and *cnf2* genes. All of the NTEC strains had the *eae* gene and most of them also carried the *ehxA* gene. Moreover, all the *cnf1*$^-$ *cnf2*$^-$ NTEC strains were negative for several virulence markers associated with CNF1$^+$ or CNF2$^+$ strains. The *cnf* gene present in one of these strains was sequenced and analysis of the gene product revealed a new type of CNF, which was named CNF3 (and the coding gene *cnf3*). Oligonucleotide primers were designed to PCR-amplify a fragment of *cnf3*. The results showed that all strains examined in this study, except one *cnf1*$^+$strain, were *cnf3*$^+$. The association of *cnf3* with *eae* and *ehxA* suggests that *cnf3*$^+$ NTEC strains might be pathogenic for humans. [**Int Microbiol** 2007; 10(1):47-55]

Key words: necrotoxigenic *E. coli* · *cnf3*, *eae* and *ehxA* genes · sheep and goats

Introduction

Necrotoxigenic *Escherichia coli* (NTEC) strains are characterized by the production of two types of cytotoxic necrotizing factors, CNF1 and CNF2 [14]. Strains producing CNF1 have been incriminated in extra-intestinal infections in humans [1,10], while those producing CNF2$^+$ have been isolated from healthy and diarrheic cattle [6,22,23,25,26], although their participation in causing diarrhea in cattle has not been clearly established. CNFs are closely associated with specific virulence markers, an association that corresponds to some degree with genetic linkage. Thus, CNF1 is closely associated with α-hemolysin [4,9,10] and cytolethal distending toxins (CDT)-I and IV [13,31], and CNF2 with F17-b fimbriae [17,26] and CDT-III [13,28]. Moreover, two atypical NTEC strains that we previously identified in diarrheic calves negative for the *cnf1* and *cnf2* genes carried the *eae* and *ehxA* genes [22] (JA Orden, unpublished data). Although experiments have shown that CNFs produce toxic effects in lambs [15], the role of these toxins in the pathogenesis of *E. coli* infections in small ruminants is still uncertain due to, among several factors, the limited number of studies carried out on NTEC strains from sheep and goats. To our knowledge, ours has been the only group to study the occurrence of NTEC in diarrheic lambs and goat kids [5,11], and no studies on the occurrence of NTEC in healthy sheep and

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goats have been published. Thus, the present study was designed to determine the occurrence and characteristics of NTEC strains in diarrheic and healthy small ruminants.

Materials and methods

Specimen collection and *E. coli* strains. Fecal samples were collected from 129 diarrheic lambs during 32 outbreaks of neonatal diarrhea and from 258 non-diarrheic sheep (116 lambs and 142 adults) on 11 farms. Likewise, samples were also collected from 17 diarrheic goat kids during seven outbreaks of neonatal diarrhea, and from 253 non-diarrheic goats (94 goat kids and 159 adults) on ten farms. Samples were collected directly from the rectum in sterile plastic bottles and shipped to the laboratory by express mail on the day of sampling. The lambs and goat kids included in this study were up to 4 weeks of age, while adults were older than 6 months. The farms participating in the study were located in the central region of Spain.

Fecal samples were plated on MacConkey agar. After an overnight incubation, four isolates with the typical appearance of *E. coli* were randomly chosen from each sample. Altogether, 2628 isolates were identified as *E. coli* by biochemical tests, including hydrogen sulfide, citrate, urease, and indole. These isolates were stored at room temperature in nutrient broth with 0.75% agar. In this work, four *eae*$^+$ NTEC strains previously isolated from two diarrheic calves and two diarrheic goat kids [11,12,22] were included in the study. The strains from calves (one belonging to serotype O153:H11 and another to serotype O111:H$^-$) were considered atypical NTEC because they produced CNF in tissue-culture assays but were negative by PCR for both *cnf1* and the *cnf2* [22], while the goat kids strains (both belonging to serotype O153:H$^-$) were considered as CNF2 producers only on the basis of biological tests [11,12].

Conditions of the experiments. Production and detection of CNF in Vero and HeLa cells were carried out as described previously [22]. Strain *E. coli* BM2-1 (CNF) was used as the positive control.

For detection of CDT, nonconfluent HeLa cells in eight-well chamber slides were infected with bacterial cultures. At specified times, morphological and cytoskeletal changes were evaluated by means of an immunoperoxidase technique that allows simultaneous visualization of the cytoskeleton and nuclei [28]. *E. coli* BM2-1 (CDT) was used as the positive control.

Detection of *cnf1*, *cnf2*, and *ehxA* by PCR was carried out as described in earlier studies [22,29]. Strains *E. coli* BM2-1 (*cnf1*), 711$^+$ (*cnf2*), and FV900a (*ehxA*) served as positive controls.

Detection of F17 fimbriae by slide agglutination was carried out as previously described [22]. Strains *E. coli* A_{11a2} and HS served as positive and negative controls, respectively.

For detection of α-hemolysin, bacteria were grown for 20 h at 37ºC on blood agar base medium (Difco) containing 5% (v/v) washed sheep erythrocytes. *E. coli* BM2-1 (α-hemolysin) was used as the positive control.

Detection of *eae* and its types. All of the NTEC isolates were analyzed by PCR for the presence of *eae* and determination of its types, as described previously [7,8]. *E. coli* strains used as controls were: EPEC-2348 (*eaeα1*), AEEC-IH2498a (*eaeα2*), EPEC-337 (*eaeβ1*), EPEC-359 (*eaeξR/β2B*), EPEC-BL152.1 (*eaeδ/β2O*), AEEC-6044/95 (*eaeκ*), STEC-EDL933 (*eaeγ1*), STEC-TW07926 (*eaeθ/γ2*), STEC-VTB-286 (*eaeε1*), AEEC-IH3205a (*eaevR/ε2*), STEC-VTO-50 (*eaeζ*), AEEC-CF11201 (*eaeη1*), H03/53199a (*eaeη2*), AEEC-7476/96 (*eaeι1*), AEEC-217-2 (*eaeμR/ι2*), AEEC-68-4 (*eaeλ*), EPEC-373 (*eaeμB*), AEEC-IH1229a (*eaevB*), STEC-B49 (*eaeξB*), AEEC-IH2997f (*eaeo*), and K12-185 (negative for *eae*).

Serotyping. O and H antigens were identified as described by Guinée et al. [19], employing all available O (O1 to O185) and H (H1 to H56) antisera. The antisera were absorbed with the corresponding cross-reacting antigens to remove nonspecific agglutinins. O antisera were produced in the *E. coli* Reference Laboratory (LREC), University of Santiago de Compostela [Lugo, Spain, http://www.lugo.usc.es/coli], and H antisera were obtained from the Statens Seruminstitut (Copenhagen, Denmark).

General DNA techniques and inverse PCR. Restriction enzymes were purchased from New England BioLabs and used according to the manufacturer's instructions. Plasmid DNA was extracted using the Plasmid Purification kit from Qiagen. Chromosomal DNA was extracted and purified from the GFX Genomic Blood DNA Purification Kit from Amersham. PCR was carried out using HotMaster *Taq* DNA Polymerase from Eppendorf. The PCR reaction mixes contained (for a volume of 100 µl) 50 ng DNA template (except for inverse PCR, see below), 4 µl of 10 mM dNTP mix from Applied Biosystems, 10 pM of each oligonucleotide primer, the suitable amount of 10× *Taq* buffer with autoadjustable Mg^{2+}, and 2 U of polymerase per kb. The standard amplification programme was: 5 min at 94ºC; 30 cycles of 1 min at 94ºC, 30–60 s at 42–55ºC, 1–3 min at 68ºC; and 10 min (1 h when the product was to be used for cloning) at 68ºC. PCR products were purified with the SpinPrep PCR Clean-up or Gel DNA kit (when agarose purification was required), purchased from Novagen.

Genome walking was carried out by successive inverse PCRs. Chromosomal DNA samples (10 µg) were digested in a reaction volume of 100 µl containing several restriction enzymes (*Bam*HI, *Bsr*GI, *Acu*I, *Bgl*II, *Bcl*I, *Eco*RI, *Hin*dIII, *Xba*I, *Sac*I) at 37ºC overnight and precipitated with Colour paint coprecipitant (Novagen) according to the manufacturer's instructions. The precipitate was resuspended and ligated with 2000 U of T4 ligase (New England BioLabs) at 16ºC overnight in a 100-µl reaction volume. The ligation mixture was precipitated as above and resuspended in 30 µl of H_2O MilliQ. Four-µl samples were used for PCRs, which was done as described above except that the elongation step was 3 min at 68ºC. DNA was sequenced at the Sequencing Unit of CIB-CSIC (Madrid, Spain) and SECUGEN SL. All sequences were determined on both strands and confirmed using products directly obtained from three independent amplifications of genomic DNA preparations. DNA sequences were assembled using the Vector NTI suite 9.0.0 and homology searches were carried out with BLAST at the National Center for Biotechnology Information, Bethesda, USA [http://www.ncbi.nlm.nih.gov/BLAST]. Multiple alignments were generated with the CLUSTALW program.

PCR amplification of *cnf3*. On the basis of the nucleotide sequence of *cnf3*, the specific primers CNF3-3D (5´-TAACGTAATTAGCAAAGA-3´) and CNF3-4R (5´-GCTGGAAAAATTAGATGAG-3´) were designed. All NTEC strains identified in this study, as well as the four previously described *eae*$^+$ NTEC strains obtained from diarrheic goat kids and calves [11,12,22], were analyzed with this PCR approach and using total DNA as template. In addition, a collection of 50 *cnf1*$^+$ or *cnf2*$^+$ NTEC strains (25 and 25, respectively) was used to validate the PCR results. These *cnf1*$^+$ and *cnf2*$^+$ strains were *eae*$^-$ and were isolated from humans and cattle [1,4,22,23].

Pulsed-field gel electrophoresis. PFGE was done in a CHEF MAPPER system (BioRad, Hemel Hempstead, United Kingdom) at 14ºC in 0.5×TBE using a standard protocol [http://www.foodborne-net.de/content/e25/e70/e580/index_ger.html]. The agarose-embedded DNA was cleaved with 0.2–0.8 U *Xba*I/µl (Roche) according to the manufacturer's instructions. Run times and pulse times were 2.20–54.0 s for 22 h with linear ramping. PFGE was used to establish clonal relatedness and diversity among a group of 33 *eaeβ1*$^+$ *E. coli* strains: 17 *cnf3*$^+$ strains and 16 O177:H11/H$^-$ *cnf3*$^-$ strains, of which 12 produced verotoxins (VTs). Of the 17 *cnf3*$^+$ strains studied by PFGE, 12 were detected in this study, four were described in previous work [11,12,22], and one had not been previously described (VTO-163) [J Blanco, unpublished data]. The 16 *cnf3*$^-$ strains were isolated from the Galicia (northwestern) and Murcia (southeastern) regions of Spain and belonged to a serotype also detected among *cnf3*$^+$ strains. They were therefore included in the PFGE analysis in order to compare their genetic rela-

tionships with the strains under study. The PFGE pulsotypes were compared by analyzing TIFF files with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis of the Dice similarity indices based on the unweighted pair group method using arithmetic averages (UPGMA) was used to generate a dendrogram describing the relationship among pulsotypes. A difference in the patterns of at least one restriction fragment was considered the criterion for discriminating between clones.

Nucleotide sequence accession number. The *cnf3* region sequence reported here from *E. coli* strain C48a was deposited in GenBank (NCBI) under accession number AM263062.

Results

Occurrence of NTEC and detection of *cnf1* and *cnf2* in NTEC strains. CNF was detected in 54 isolates from 18 animals. Table 1 shows the occurrence of NTEC in the different groups of sheep and goats studied. All NTEC isolates were investigated for the presence of *cnf1*, *cnf2*, *ehxA*, and *eae* (and their types), production of CDT, F17 fimbriae, and α-hemolysin, and serotype. When the NTEC isolates from an animal showed the same characteristics it was assumed that they were the same strain. In all sheep and goats infected with NTEC, only one strain per animal was identified. Thus, a total of 18 NTEC strains were obtained by tissue-culture assays (Table 2). Four (22.2%) of the NTEC strains were positive for *cnf1*, as determined by PCR, while the remaining 14 (77.8%) were negative for both *cnf1* and *cnf2*. Moreover, PCR assays showed that the two *eae*$^+$ NTEC strains isolated from diarrheic goat kids in previous works [11,12] and considered CNF2 producers according to biological tests were negative for *cnf1* and *cnf2*.

Association between *cnf* and virulence markers. None of the NTEC strains in the present study was positive for F17 antigen or CDT and only one produced α-hemolysin (Table 2). In contrast, all the NTEC strains were positive for *eae* and, except three strains, also for *ehxA*. All NTEC strains tested, except one, possessed *eaeβ1*.

Cloning and sequencing of *cnf* from NTEC strain C48a (O153:H11). Since most of the NTEC strains isolated from sheep and goats were negative for *cnf1* and *cnf2* as well as for some virulence markers associated with *E. coli* strains producing CNF1 and CNF2, the existence of an as-yet-unknown type of CNF was suspected. We therefore decided to clone and sequencing the *cnf* gene present in NTEC strain C48a (O153:H11), isolated from healthy sheep. The nucleotide sequences of *E. coli cnf1* and *cnf2* (accession numbers X70670 and U01097, respectively) [18,27] were used in a multiple nucleotide sequence alignment, and regions with high homology were selected in order to design several primer combinations. Among those primers, only CNF3-1Dbis (5´-ATATCTTCTTGAGTACAATGA-3´) and CNF3-1R (5´-ATTACCACGATTTAATTTCTG-3´) produced a positive specific, although faint, amplification of a 2,182-bp fragment in a PCR (annealing temperature of 30ºC) with total DNA from *E. coli* C48a. This fragment was inserted into the pCR 2.1-TOPO cloning vector (Invitrogen) and sequenced. The new sequence was extended by genome walking using inverse PCR with total DNA from *E. coli* C48a (see Materials and methods) and specific primers based on the newly determined nucleotide sequence.

The first positive inverse PCR amplicon (910-bp) was obtained with *Bgl*II and the specific primers combination CNF3-3R (5´-TCTTTGCTAATTACGTTA-3´)/CNF3-4D (5´-CTCATCTAATTTTTCCAGC-3´); the second inverse PCR (1,744-pb) was generated with *Bsr*GI and CNF3-2R (5´-GCGGAACAGACACTTCCGG-3´)/CNF3-4D (5´-CTC ATCTAATTTTTCCAGC-3´); and the third inverse PCR fragment (3,758-pb) with *Hin*dIII and CNF3-10R (5´-TCAACCCTACTAAACCAG-3´)/CNF3-9D (5´-ATGATCG GTATTTCTATA-3´). Overlapping the three inverse PCR amplicons yielded a region of 4,320 bp (GenBank accession number AM263062). Analysis of this sequence identified a large open reading frame (ORF) of 3,042 nucleotides with a predicted translation product of 1,013 amino acids that was highly similar to CNF1 (70.1% identity) and CNF2 (69.9% identity). The newly identified gene was named *cnf3* and its encoded product CNF3. The homology between CNF3, CNF1, and CNF2 is evenly distributed throughout the entire sequence. However, two large areas of dissimilarity, 17 and four amino acids in length (positions 411–427 and 565–568) are shared by the three *E. coli* CNFs. Furthermore, CNF3 showed four exclusive areas of dissimilarity, spanning the

Table 1. Occurrence of NTEC in the different groups of sheep and goats studied

Species and group of animals	No. animals infected with NTEC /No. examined (%)
Sheep	
Healthy sheep	14/258 (5.4%)
Lambs	13/116 (11.2%)
Adults	1/142 (0.7%)
Diarrheic lambs	1/129 (0.8%)
Goats	
Healthy goats	3/253 (1.2%)
Goat kids	3/94 (3.2%)
Adults	0/159 (0%)
Diarrheic goat kids	0/17 (0%)

Table 2. Serotypes and virulence markers in NTEC strains

Source and serotype	No. of NTEC strains	*cnf* type	*eae* type	α-hemolysin	*ehxA*
Strains described in the present study					
Healthy lambs					
O4:H$^-$	1	1	β1	+	+
O4:H$^-$	2	1, 3	β1	–	+
O76:H$^-$	1	3	ε1	–	+
O153:H11	5	3	β1	–	+
O153:H11	1	3	β1	–	–
O177:H11	1	3	β1	–	+
O177:H11	1	3	β1	–	–
ONT:H11*	1	3	β1	–	+
Healthy adult sheep					
O177:H11	1	3	β1	–	+
Diarrheic lambs					
O26:H$^-$	1	3	β1	–	+
Healthy goat kids					
O4:H11	1	1, 3	β1	–	+
O4:H11	1	3	β1	–	+
O4:H11	1	3	β1	–	–
Strains described previously					
Diarrheic calves [22]					
O111:H$^-$	1	3	β1	–	+
O153:H11	1	3	β1	–	+
Diarrheic goat kids [11,12]					
O153:H$^-$	2	3	β1	–	+

*Not-typable strain.

regions 174–179, 580–587, 765–770, and 904–917 (Fig. 1). Cysteine and histidine residues essential for the biological activity of CNF1 and CNF2 [33] were conserved in the predicted sequence of CNF3. Computer analysis of its deduced amino acid sequence showed a putative membrane protein with a calculated molecular mass of 114,486 kDa, an isoelectric point of 4.89, and the absence of a consensus signal sequence in the N-terminal. In silico study of the protein in a search for transmembrane helix domains, topology, and subcellular localization (TMHMM2, SOSUI, TMpred) resulted in the classification of CNF3 as an inner-membrane protein containing two putative transmembrane domains (amino acids 352–372 and 386–406).

In silico analysis of the *cnf3* DNA boundaries revealed significant similarities to mobile genetic elements, such as transposases and insertion sequences. The overall content of guanosine and cytosine (percent G + C) in the DNA fragment sequenced in this study was 36.3%, but there was a remarkable difference within the gene, i.e., the percent G + C upstream of *cnf3* was 37.4% while that of the sequence comprising the 3´end was 46%.

Detection of *cnf3* by PCR. Amplification products of the expected size (1,246 bp) were detected in all NTEC strains examined, except one *cnf1*$^+$ strain, as well as in the four *eae*$^+$ NTEC strains previously described in diarrheic goat kids and calves [11,12,22]. In contrast, no amplification products were seen in a PCR with DNA obtained from the 50 *cnf1*$^+$ or *cnf2*$^+$ NTEC strains of our collection isolated from humans and cattle [1,4,22,23]. Therefore, the specificity and sensitivity of the pair of oligonucleotide primers designed in this study for detecting *cnf3* was 100%.

Serotyping. The *cnf3*$^+$ *E. coli* strains isolated from sheep and goats belonged to seven different serotypes (Table 2). The most frequent serotypes among these strains were O4:H11, O153:H11, and O177:H11. The *cnf1*$^+$ *cnf3*$^-$ NTEC strain belonged to serotype O4:H$^-$.

PFGE patterns. Thirty-three strains were analyzed by PFGE: 13 O177:H11, seven O177:H$^-$, seven 153:H11, two O153:H$^-$, two O4:H11, one O111:H$^-$, and one ONT:H11.

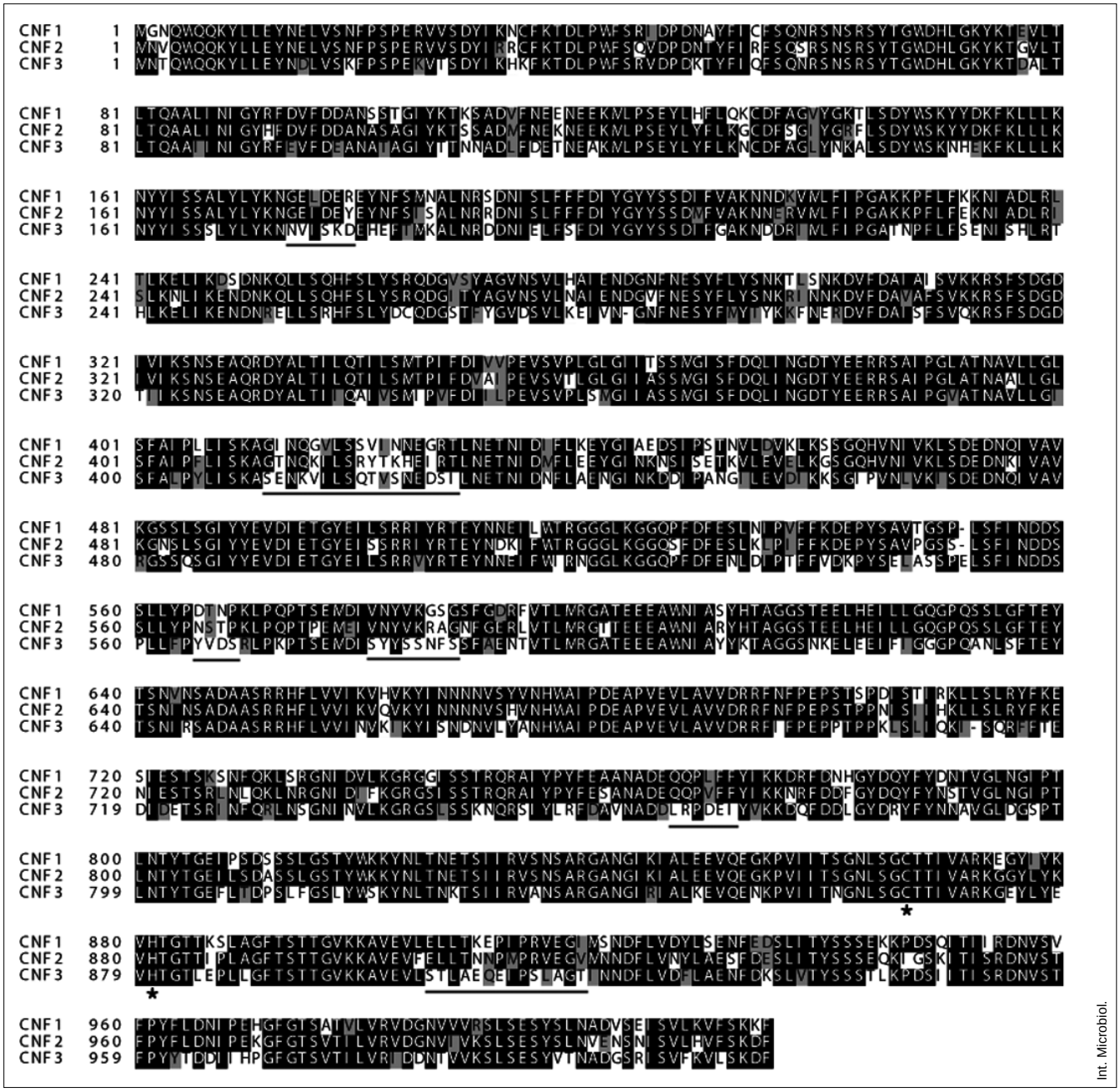

Fig. 1. Multiple amino-acid sequence alignment of the predicted amino-acid sequence from the three *Escherichia coli* CNF types. Identical residues are shaded black with white letters, similar residues gray with black letters. Asterisks indicate the conserved cysteine and histidine essential for CNF activity [30]. Main areas of dissimilarity are underlined. Multiple amino-acid sequence alignment was generated with the AlignX tool of the Vector NTI 9.0 software and displayed graphically using BOXSHADE 3.21.

Genomic DNA fingerprinting revealed 31 distinct *Xba*I restriction patterns, based on a difference of at least one restriction fragment in the patterns as the criterion for discriminating between them. According to the dendrogram produced by the UPGMA algorithm, the strains could be classified into four groups (I–IV) of >70% similarity according to the Dice similarity index (Fig. 2).

Analysis of each of these four groups showed that group I clustered all seven O177:H$^-$ *vt2*$^+$ *cnf*$^-$ strains, and six subgroups with similarity >85% could be differentiated (I-A: two strains; I-B, I-C, I-D, I-E, and I-F with one strain each). Strains of serotype O177:H11 clearly clustered in two different groups (II and IV). All five O177:H11 strains clustering in group IV were *vt1*$^+$ *cnf*$^-$, with similarity >85%. The eight

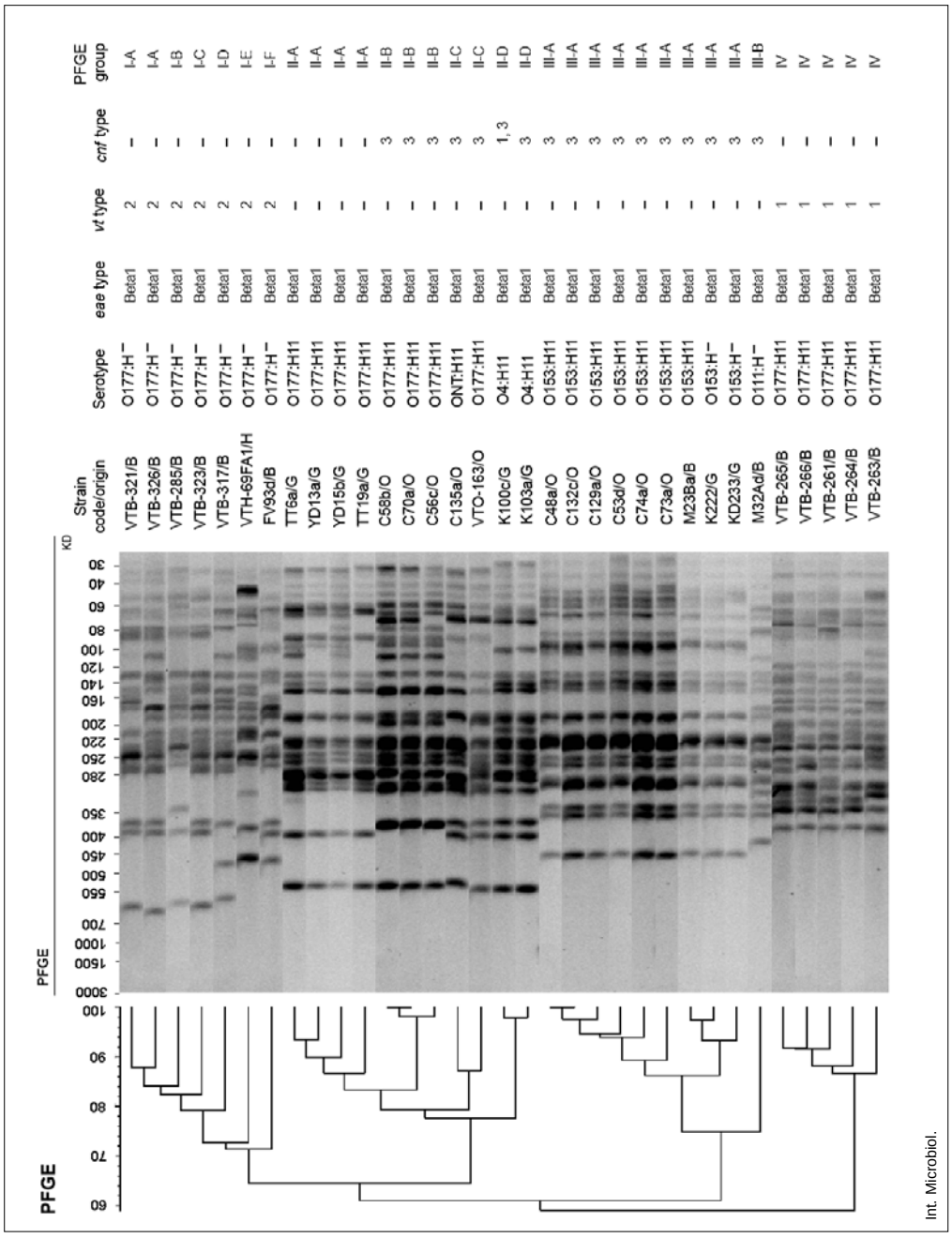

Fig 2. Dendrogram generated by Bionumeric software, showing distance calculated by the Dice similarity index of pulsed-field gel electrophoresis *Xba*I patterns among 33 *E. coli* strains from different origins (B, bovine; H, human; G, goat; O, ovine). The degree of similarity (%) is shown.

O177:H11 strains of group II were *vt*$^-$ *cnf*$^-$ or *vt*$^-$ *cnf3*$^+$ and consisted of three small subgroups (>85% similarity): II-A, with four *vt*$^-$ *cnf*$^-$ strains; II-B, with three *vt*$^-$ *cnf3*$^+$ strains; and II-C, with one *vt*$^-$ *cnf3*$^+$ strain. Group III was the most homogeneous, clustering all nine O153:H11/H$^-$ *vt*$^-$ *cnf3*$^+$ strains (>85% similarity) in subgroup III-A, with one O111:H$^-$ *vt*$^-$ *cnf3*$^+$ strain in subgroup III-B.

Discussion

Cytotoxic necrotizing factors have been associated with diarrhea and other clinical signs in lambs [15]. However, the number of studies carried out on NTEC strains from sheep and goats is very limited. The study reported here describes

the occurrence of NTEC in diarrheic and healthy lambs and goat kids as well as in healthy adult sheep and goats. This is, to our knowledge, the first report of the occurrence of NTEC in these animals. Healthy lambs were more often infected with NTEC (11.2%) than healthy adult sheep (0.7%) or diarrheic lambs (0.8%). Moreover, none of the healthy adult goats or diarrheic goat kids and only three (3.2%) healthy goat kids were infected by NTEC. The proportions of diarrheic lambs and goat kids infected with NTEC (0.8% and 0%, respectively) were slightly lower than reported previously (1.4 and 3.6%, respectively) [5,11]. Thus, our results suggest that NTECs are not associated with neonatal diarrhea in small ruminants. However, NTECs should not be overlooked, because, as suggested by Van Bost et al. [33], they may represent opportunistic pathogens waiting for favorable circumstances.

Analysis of the sequence from the newly identified gene from NTEC strain C48a identified an ORF with a predicted translation product that was highly similar to CNF1 and CNF2 (70.1 and 69.9% identity, respectively). This gene was subsequently named *cnf3* and its encoded product CNF3. Cysteine and histidine residues essential for the biological activity of CNF1 and CNF2 [30] are conserved in the predicted sequence of CNF3.

In silico analysis of CNF3 showed that it is an inner-membrane protein containing two putative transmembrane domains. These features are shared with CNF2 [27]. Analysis of the *cnf3* boundaries revealed the presence of mobile genetic elements, suggesting the mobility of *cnf3*. Moreover, it points to the instability inherent in any evolutionarily new DNA carrying insertion sequences in the absence of in vivo selective pressures for conservation. Moreover, the percent G + C in the DNA fragment sequenced was similar to the percentages reported for *cnf1* [18] and *cnf2* [27] in *E. coli*, and *cnfy* in *Yersinia pseudotuberculosis* [20].

On the basis of the nucleotide sequence of *cnf3*, oligonucleotides were designed to amplify a fragment of the gene by PCR. Our results showed that the specificity and sensitivity of the primer pair designed in this study was 100%. According to the results obtained with the specific PCR assay designed for detecting *cnf3*, all NTEC strains isolated in this study, except one, as well as the four *eae*$^+$ NTEC strains described in previous works [11,12,22], carried the gene. The two *eae*$^+$ NTEC strains isolated from diarrheic goat kids were previously classified incorrectly as CNF2 producers because the CNF type was identified by biological assays only [11] and, as we described previously [22], CNF2 and CNF3 produce similar effects in rabbits and mice.

Three of the 17 (17.6%) NTEC strains isolated from the animals studied carried *cnf1* and *cnf3*, also had *eae* and *ehxA*, and belonged to serotypes O4:H$^-$ (2 strains) and O4:H11. Moreover, one of the NTEC strains carried *cnf1* but not *cnf3*. This strain possessed *eae* and *ehxA*, produced α-hemolysin, and belonged to serotype O4:H$^-$. CNF1 and α-hemolysin are closely associated [1,9,10], and serogroup O4 is one of the most prevalent among NTEC producing CNF1 [16,21]. However, to our knowledge, ours is the first description of *cnf1*$^+$ NTEC strains that also have *eae* or *ehxA*. None of the *cnf3*$^+$ *cnf1*$^-$ NTEC strains produced α-hemolysin (a virulence marker of CNF1$^+$ strains) [1,9,10], expressed F17 fimbriae (a virulence marker of CNF2$^+$ strains) [17,26], or produced CDT (a virulence marker of CNF1$^+$ and CNF2$^+$ strains) [13,28,31]. All *cnf3*$^+$ *cnf1*$^-$ NTEC strains possessed *eae* and, except in three strains, also carried *ehxA*. In contrast, CNF1$^+$ or CNF2$^+$ strains are rarely associated with *eae* and *ehxA* [23,25]. The most frequent serotypes of the *cnf3*$^+$ NTEC strains were O4:H11, O153:H11, and O177:H11, which have not been previously identified in CNF1$^+$ and CNF2$^+$ strains [2,3].

Since the *cnf3*$^+$ strains found previously [11,12,22] and in this work were *eae*$^+$, *bfpA*$^-$ (data not shown), *vt*$^-$, and, in addition to producing CNF, almost all had *ehxA*, it is possible that these strains are atypical enteropathogenic *E. coli* (EPEC) and thus potentially pathogenic for humans [32]. By analogy with human strains, *eae*$^+$ *vt*$^-$ *E. coli* strains isolated from animals are usually referred to as EPEC, preceded by the animal type from which the strains were isolated (i.e., ovine EPEC).

As all *cnf3*$^+$ strains possessed *eae*, we propose calling such strains necroenteropathogenic *E. coli*. On the basis of antigenic variation, PCR analysis, and sequencing, at least 21 types of *eae* have been identified [7]. Except one, all *cnf3*$^+$ *eae*$^+$ strains identified thus far, including the strains described previously [11,12,22], had *eae* type β1. The exception was a strain isolated from a healthy lamb and belonging to serotype O76:H$^-$, which had *eae* type ε1. Type β seems to be the most widespread type among human and animal *eae*$^+$ *E. coli* strains, while type ε is frequently found in *eae*$^+$ *E. coli* strains from healthy sheep and goats [24].

As was the case in a previous study [8], strains of the same serotype grouped closely in the dendrogram. The 17 *cnf3*$^+$ strains analyzed by PFGE comprised five clusters (>85% similarity): II-B (O177:H11), II-C (O177:H11 and ONT:H11), II-D (O4:H11), III-A (O153:H11/H$^-$), and III-B (O111:H$^-$). Strains belonging to serogroup O153 were more homogeneous than strains of serogroup O177. Interestingly, O177:H11 *cnf3*$^+$ *eaeβ1*$^+$ strains clustered in groups or subgroups different than those of O177:H11/H$^-$ *cnf*$^-$ *eaeβ1*$^+$ from enterohemorrhagic *E. coli* (*eae*$^+$ *vt*$^+$ *E. coli* strains) and atypical EPEC (all *eae*$^+$ *vt*$^-$ *E. coli* strains analyzed by PFGE were *bfpA*$^-$) pathotypes.

Taken together, the data on NTEC show that the characteristics of *cnf3*$^+$ strains are generally different than those of CNF1$^+$ and CNF2$^+$ strains: (i) most of the NTEC isolated from sheep and goats are *cnf3*$^+$, whereas NTEC from humans, pigs, cats, and dogs are CNF1$^+$ and most NTEC from cattle are CNF2$^+$ [2,3,16,21, this study]; (ii) all *cnf3*$^+$ *E. coli* strains have *eae* and most of them also carry *ehxA*, whereas CNF1$^+$ or CNF2$^+$ strains are rarely associated with *eae* and *ehxA* [22,23,25, this study]; (iii) none of the *cnf3*$^+$ NTEC strains was positive for several of the virulence markers (CDT, α-hemolysin, F17 fimbriae) classically associated with CNF1$^+$ and CNF2$^+$ strains [22, this study]; and (iv) the most prevalent serotypes of the *cnf3*$^+$ NTEC strains were O4:H11, O153:H11, and O177:H11, which have not been previously found in CNF1$^+$ and CNF2$^+$ strains [2,3; this study]. Moreover, our data [11,12,22, this study] show that the *cnf3*$^+$ NTEC strains can be frequently isolated from a representative percentage of healthy lambs but only sporadically from healthy adult sheep and goat kids and from diarrheic calves, lambs, and goat kids.

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