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Occurrence and functionality of cycle inhibiting factor, cytotoxic necrotising factors and cytolethal distending toxins in *Escherichia coli* isolated from calves and dogs in Italy

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Abstract.

Escherichia coli isolated from animals up to three months of age, with diarrhea (255 calves and 29 dogs (pups)), without diarrhea (21 calves and 11 pups, used as controls), and 58 adult dogs with cystitis were tested to investigate the occurrence and functional expression of cyclomodulins cycle inhibiting factor (CIF), cytotoxic necrotizing factors (CNFs) and cytolethal distending toxins (CDTs). In cyclomodulin-positive isolates the association was assessed with other virulence genotypes and phylogenetic groups. Of 374 *E. coli* isolates, 80 (21.4%) were positive for at least one cyclomodulin and 14 of the latter (3.7%) showed different combinations of more than one. cif-positive isolates showed a low number of additional virulence factors, and were commonly associated with phylogroup B1, while *cnf*- and cdt-positive isolates, harboring many extraintestinal virulence factors, belonged to phylogroups B2 and D. Almost all isolates showed an irreversible cytopathic effect (CPE), displaying functionality of cyclomodulins. Five isolates that presented a mutation of *cif* were CPE-negative.

Introduction

Escherichia coli of biological significance can be broadly classified into three major groups: commensals or nonpathogenic, intestinal pathogenic and extraintestinal pathogenic (Russo and Johnson, 2000). The ability of *E. coli* to colonize intestinal and extraintestinal sites is partly due to its versatile genome, via the production of a broad array of virulence factors which manipulate basic host cell functions, such as the cyclomodulins, a growing family of bacterial toxins and effectors, which interfere with the eukaryotic cell-cycle. Examples are inhibitory cyclomodulins, such as the cycle inhibiting factor (CIF), cytolethal distending toxins (CDTs) and cyclomodulins that promote cellular proliferation, such as cytotoxic necrotizing factors (CNFs) (Nougayrède et al., 2005). A fourth toxin, colibactin, encoded by the genomic island *pks*, has been investigated, and this protein seems to act mainly as a colonization factor, rather than a virulence factor (Dubois et al., 2010). The *cif* gene is found mainly in enteropathogenic and enterohaemorrhagic *E. coli* (EPEC and EHEC, respectively), while *cdt* and *cnf* genes can be found both in intestinal and extraintestinal isolates. In cultured cells, *E. coli* producing CIF, triggers an irreversible cytopathic effect (CPE) characterized by the progressive recruitment of focal adhesions, assembly of stress fibersand arrest of the cell cycle at the G2/M phase. Studies have shown that some mutations (deletion, substitution, addition of a nucleotide or insertion of a partial insertion sequence 2 (IS2)) of the *cif* gene can lead to a non-functional protein (Marchès et al., 2003; Loukiadis et al., 2008). Two main types of CNF proteins have been described, CNF1 and CNF2. CNF1-producing *E. coli* are involved in extraintestinal or intestinal diseases in humans and animals, and CNF2-producing *E. coli* are associated mainly with enteritis or bacteraemia in calves and lambs (Dozois et al., 1997). The CNF toxins are able to induce the formation of giant multinucleated cells in tissue culture (De Rycke et al., 1990). The CDTs are a family of toxins which have not been associated with any particular pathotype of *E. coli*, except for a few isolates of EPEC serogroups (Oloomi and Bouzari, 2008). Three adjacent genes *(cdtA, cdtB* and *cdtC)* are required for protein expression and five CDTs (CDT-I to CDT-V) have so far been reported (Toth et al., 2009). The CDTs induce a progressive cellular distension and death of various cell lines cultivated *in vitro*, but the actual effect of these toxins remains unclear since CDTs have not played a significant role in acute infection models tested to date (Young et al., 2001; Van Bost et al., 2003b). Additional virulence factors like hemolysin (hly), aerobactin *(aer)*, P-fimbriae *(pap)*, S-fimbriae *(sfa)*, type 1 fimbriae *(fimA)* and afimbrial adhesin *(afa)* have been described mainly in extraintestinal *E. coli* (Johnson et al., 2001). Moreover, intimin *(eae)*, shiga-toxins (stx1 and *stx2)*, and heat stable toxins (st1 and *st2)* are typical virulence factors associatedwith pathogenic intestinal *E. coli* (Kaper et al., 2004). *E. coli* populations have a clonal structure, and indeed, various intestinal and extraintestinal *E. coli* infections have been linked to specific clones or groups of related clones. Phylogenetic studies have shown that *E. coli* can be divided into four main groups designated A, B1, B2 and D. Pathogenic *E. coli* responsible for diarrhea are found predominantly in groups A or B1 (Escobar-Paramo et al., 2004), while most *E. coli* responsible for urinary tract infections and other extraintestinal infections belong to group B2 or to a lesser extent group D, while commensal *E. coli* are found predominantly in groups A, B1 and D (Duriez et al., 2001).

Diarrheagenic *E. coli* have been associated with enteric diseases in young calves and dogs, while uropathogenic isolates have been closely correlated with urinary infections in dogs, particularly adults and aged dogs. Previous studies have suggested that cyclomodulin-positive strains were isolated from both intestinal and extraintestinal *E. coli* strains. The aim of this study was to investigate the occurrence of cif, *cnf* and *cdt* genes in *E. coli* isolates from calves and dogs (pups) up to three months of age, with and without diarrhea, and from adult dogs with cystitis. Both the functional expression and the association with other virulence genotypes and phylogenetic groups were assessed in cyclomodulin-positive isolates.

Materials and methods

Bacterial isolates. Three hundred and seventy-four *E. coli* were isolated from fecal samples collected from animals (up to three months of age) with diarrhea (255 calves and 29 pups), without diarrhea (21 calves and 11 pups, used as controls), and from urine collected from 58 adult dogs (from 2 to 12 years; mean age seven years) with cystitis. All samples were collected from animals in North West Italy (NW Italy) between November 2007 and April 2010. Bovine and canine feces were swabbed directly from the rectum, and urine samples were collected by cystocentesis. Samples were cultured on Mac-Conkey agar (Oxoid, Besingstoke, England) and lactose-fermenting, indole-positive colonies were evaluated by the BBL Crystal test (Becton, Dickinson, Co., USA). All *E. coli* isolates (one colony per sample) were stored in Luria-Bertani broth (Oxoid) containing 15% glycerol at -80 °C until use. The genomic DNA of the bacteria was extracted by using InstaGene™ DNA extraction Kit (Bio-Rad Laboratories, Canada).
Detection of cyclomodulins in E. coli isolates. PCRs were used to investigate *cif, cnf* and *cdt* genes from all *E. coli* isolates. Primer sets are listed in Table 1. The *cif* gene was assessed using internal primers while the other two primers were used in positive isolates to detect if the *cif* gene was complete (Marchès et al., 2003). The presence of a conserved region of the *cnf* gene (Van Bost et al., 2003a) was also investigated and the two major classes of *cnf* genes (cnf1 and *cnf2)* were recognized from positive samples (Pass et al., 2000). To determine the presence of *cdt,* two pairs of primers for the consensus region of *cdtB* gene were used in a multiplex PCR. On the basis of existing sequence differences in the *cdtB* genes, type-specific primers were used for typing the *cdt* genes (Toth et al., 2003; Bielaszewska et al., 2004).
All PCRs were performed in a volume of 50 lL with a reaction mix containing 20 pmol of each primer added to 5 µl of reaction buffer 10x, 200 µmol dNTPs mix (Qiagen, Gmbh, Hilden, Germany), 0.5 µl of Taq polymerase (Qiagen). Five microliters of extracted DNA of each isolate were added to the reaction mix. The amplification protocol included an initial denaturation of 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, different annealing temperatures (see Table 1) for 1 min and an elongation of 1 min at 72 °C. This was followed by a final extension of 5 min at 72 °C. Reaction mixtures were separated by electrophoresis on 2% agarose gel. Negative and positive controls were used in all assays.

Determination of the type of mutation in E. coli isolates with a partial cif gene. Isolates with only a partial *cif* gene were amplified and sequenced to detect the presence of mutations in the 3 -end of the gene. Two different pairs of primers mut1 and mut2 (see Table 1) were designed in this study on the basis of an alignment of published gene sequences. As positive controls, we used genomic DNA extracted from *E. coli* strains PMK5 (AY128538), H19 (AY128539), RW1374 (AY128543) and E2348/69 (AF536197), kindly provided by Eric Oswald (Laboratoir Associé INFRA-ENVT de Microbiologie, Toulouse, France). Mut1 primers were used to detect the presence of addition or deletion of adenine to nucleotide 818 (PMK5 and H19 strains) or to determine the substitution of a nucleotide (c/t) at 735 bp of the *cif* gene (RW1374 strain). Mut2 primers were designed to detect the insertion of a partial IS2 at nucleotide 655 bp (E2348/69 strain). PCRs were performed in a volume of 50 µl with a reaction mix containing 20 pmol of each primer added to 5 µl of reaction buffer 10x, 200 µl mol dNTPs mix (Qiagen), 0.5 µl of Taq polymerase (Qiagen). Five microliters of extracted DNA of each isolate were added to the reaction mix. The amplification program consisted of 35 cycles of 1 min at 94 °C, different annealing temperatures (see Table 1) for 1 min and 1 min at 72 °C, followed by an extension of 5 min at 72 °C Amplicons were purified using the QIAquick PCR purification kit (Qiagen) and sequenced to confirm the presence of mutations. PCR product sequencing was performed using PCR-derived primers in ABI Prism 310 Genetic Analyzer (Applied Biosystems, Milan, Italy) by the dideoxy chain termination method with fluorescent dye terminators. The sequences obtained were analyzed using the CHROMAS 2.0 software (Technelysium, Helensvale, Australia) and submitted to BLAST analysis (BLAST, NCBI, USA). Sequences were then aligned using ClustalW multiple-alignment software provided in the BioEdit package, version 7.0.5.2 (Hall, 1999).

Detection of additional virulence factors. Individual PCR reactions (Blanco et al., 1997; Pass et al., 2000; Moulin-Schouleur et al., 2007) were used on cyclomodulin-posi-tive isolates to detect the occurrence of additional virulence markers: stx1, *stx2,* st1, *st2, eae* (intestinal pathogenic *E. coli), hlyA, iutA, papC, sfa, fimA* and *afa* (extraintestinal pathogenic *E. coli).*

Cytopathic effect (CPE) on epithelial cells. The CPE of isolates was used to assess the functional expression of the cyclomodulins. Human epithelial HeLa cells (ATCC CCL-2) were cultivated using Sigma products, in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% fetal calf serum (FCS), L-glutamine (200 mM) and gentamicin (50 mg/mL). The cell line was incubated at 37 °C in a 5% CO_2 atmosphere. Twenty-four hours before interaction, the cells were trypsinized and seeded in 96-well tissue culture plates to give 5 x 10^4 cells per well in a 100 µl L volume of D-MEM. The day before interaction, bacteria were cultivated overnight in Luria-Bertani broth. Interaction was carried out in D-MEM buffered with 25 mM Hepes supplemented with 5% FCS, by using a starting inoculum of 5 x 10^4 bacteria per well. After a 4 h interaction period, cells were washed three times with Hank's Buffered Salt Solution and further incubated for the CPE assay at 37 °C in a 5% CO_2 atmosphere in D-MEM with 10% FCS and gentamicin (200 µl g/mL). The morphological changes in cells were observed after a 72 h incubation period.

Determination of phylogenetic group. The phylogenetic group distribution of cyclomodulin-positive isolates was detected

by the phylogenetic grouping technique based on triplex PCR. The method uses a combination of three primer pairs *(chuA.1-chuA.2,* TspE4C2.1 -*Tsp*E4C2.2 and*yjaA.1-yjaA.2),* as described by Clermont et al. (2000). Isolates were assigned to phylogenetic groups A, B1, B2 or D according to the amplification of the *chuA* and *yiA* genes and the *TspE4C2.1* fragment.

Statistical analysis. Data were organized in 2 x 2 tables in order to explore associations between each cyclomodulin with clinical signs, virulence factors and phylogroups. In both cases, data were analyzed using the Fisher Exact probability test (FEP) to assess the significance of the associations, considering *P* values <0.05 statistically significant and P < 0.01 highly significant, respectively.

Results

Detection of cyclomodulins in E. coli isolates. The occurrence and distribution of cyclomodulins are shown in Table 2. The results obtained indicated that 21.4% of the *E. coli* isolates were cyclomodulin-positive, that is, 79 from animals with clinical signs and one from a control animal carrying the *cnf1* gene. Most positive isolates presented only one cyclomodulin (82.5%), while combinations of two cyclomodulins (17.5%) were found in six isolates, both *cif* and *cdt* positive, and eight isolates, both *cnf* and *cdt* positive. Only 15 of 20 *cif* (internal fragment)-harboring isolates contained the complete gene. Specific primers were used to type *cnf1* and cnf2, 42 were *cnf1* and three cnf2. On further characterization, cdt-positive isolates were analyzed for determination of their variant alleles. Owing to a high level of homology, *cdt-I* and cdt-IV, cdt-II and cdt-III were aggregated; 11 cdt-I/IV and 18 cdt-II/III were detected. The typing of *cnf* and *cdt* genes is not shown in Table 2.

Detection of virulence factors. The correlation between 80 cyclomodulin-positive isolates and virulence factors is reported in Table 3. In intestinal *E. coli* (47 isolated from animals with diarrhea and one from a healthy pup), frequencies of virulence factors were 58.3% for *papC,* 54.2% for *iutA,* 41.7% for *sfa,* 45.8% for *fimA,* 29.2% for *hlyA,* while *afa* and *st* toxins were not detected. All 20 cif-positive isolates were associated with the *eae* gene (P < 0.01) 16 of which were identified as EPEC (eae-positive, stx-negative) and 4 as EHEC (eae-stx-positive). In the 32 *E. coli* isolated from dogs with cystitis, the frequencies of virulence factors were *sfa* (87.5%), *papC* (71.9%), *fimA* (59.4%), *hlyA* (53.1%), *iutA* (25%) and *afa* (9.4%). The association between *cnf1* and *hly, papC, sfa* was found both among intestinal (55.6%) and urinary (50%) isolates with a highly significant result (P < 0.01).
Amplicons of the expected sizes were obtained in all five isolates with mut1 primers and the analysis of the nucleotide sequences showed a deletion of one adenine in four isolates and an addition of one adenine in one isolate. These modifications occurred in the 3^0-end of the gene (C-terminal sequence) at the nucleotide 818 (strains H19 and PMK5), corresponding to aminoacid 274, and led to a stop codon and a non-functional truncated CIF protein. In particular, the sequencing of PCR products showed a similarity of 98-100% with the former strains retrieved from the database (GenBank).

CPE on epithelial cells. All isolates harboring the complete *cif* gene, *cnf* and *cdt* showed CPE on HeLa cells. *E. coli* harboring CIF or CDT induced CPE as seen by enlarged nuclei and cell distension, while the CNF-producing isolates caused multi-nucleation and enlargement on HeLa cells. Since the CPE induced by CIF and CDT was similar, we were unable to confirm if the CPE obtained in isolates harboring both toxins was due to one or both cyclomodulins. Isolates harboring CNF and CDT produced both multi-nucleation and cell distension on HeLa cells. All isolates (n = 5) which were positive to *cif* internal fragment showed a CPE-negative phenotype.

Mutations in the partial cif gene

The determination and characterization of mutations of the *cif* gene in five CPE-negative isolates was detected using specific primers designed to feature three types of possible mutations. The correlation between cyclomodulin-positive isolates and phylogenetic groups is shown in Table 4. About 60% of the *E. coli* were in phylogenetic group B2; the remainder in B1 (15%), D (13.7%) and A (11.2%) groups. Fecal isolates from animals with diarrhea were distributed into groups B2 (40.4%), B1 (21.3%), D (21.3%) and A (17.0%). The unique isolate from the healthy dog belonged to group B2. The phylogenetic group of isolates from urine was as follows: B2 (87.5%), D (3.1%), A (3.1%) and B1 (6.2%). With intestinal isolates of *E. coli,* CIF was distributed in group B1 (64.3%), CNF in group B2 (66.6%) and CDT in group D (61.5%). Isolates with a functional CIF (n = 9) were distributed mainly in group B1 (77.8%) and to a lesser extent in groups A (22.2%), while isolates with a non-functional CIF (n = 5) were found in groups B2 (60%) and B1 (40%). *E. coli* isolates from urine that harbored CNF or CDT belonged mainly to group B2 (89.3% and 100%, respectively). The combinations CIF-phylogroup B1 and CNF1-phylogroup B2 were significant (P<0.05) and highly significant (P<0.01), respectively.

Discussion

In this study, the occurrence and the functionality of the cyclomodulins CIF, CNF and CDT in *E. coli* isolated from calves and dogs collected in Italy are described. Moreover, the association with virulence genotypes and phylogenetic groups was analyzed. At least one cyclomodulin was harbored by 21.4% of isolates, 3.7% of which showed different combinations of more than one cyclomodulin. These data are comparable to the results of other studies obtained with *E. coli* isolated from calves and humans with diarrhea (Salvadori et al., 2003; Toth et al., 2003; Loukiadis et al., 2008) and from dogs with cystitis (Johnson et al., 2003). Additionally, the low occurrence of cyclomodulins (2%) detected in fecal samples collected from healthy animals corresponds with that obtained in other studies with commensal isolates (Kadhum et al., 2008; Stenske et al., 2009; Dubois et al., 2010). Only isolates from animals with diarrhea were positive for the cifgene. The analysis of the additional virulence factors in relation to the presence of *cif* revealed that an association with *iutA* and/or *papC,* commonly

appeared in combination in animals with diarrhea. The phylogenetic analysis of cif-positive isolates showed that the majority harboring a functional CIF was in group B1 or, to a lesser extent, group A, while isolates that contained a non-functional CIF were common in group B2, in accordance with previous observations (Loukiadis et al., 2008). Regarding the *cnf* gene, isolates from the cystitis cases showed a higher occurrence of cnf1 than intestinal isolates because this factor is a typical uropathogenic virulence gene (Stenske et al., 2009). The correlation between cnf1 and additional virulence factors showed a significant association with *hlyA, papC* and *sfa* in *E. coli* isolates. The latter may be explained by their being a direct chromosomal linkage between these operons within particular DNA units on the chromosome, termed pathogenicity islands (PAIs), which carry a virulence associated gene coding for cnf1, *hlyA, papC* and *sfa* (Soto et al., 2006). The detection of this combination in isolates of intestinal origin suggests the occurrence of extraintestinal isolates that make up, at least transiently, the normal intestinal flora. The cnf1-positive isolates were firmly associated with group B2 and predictive of urosepsis origin, in accordance with Dubois et al. (2010). We found a similar percentage of the *cdt* gene both in isolates from feces (8.8%) and urine (6.9%) confirming that this cyclomodulin is produced by intestinal and extraintestinal *E. coli.* Typing of the *cdt* gene revealed *cdt-I/cdt-IV* variants among intestinal and urinary isolates from dogs and cdt-II/cdt-III among *E. coli* isolated from calves with diarrhea, in accordance with previous observations (Mainil et al., 2003). The *cdt* gene was associated with fimbrial adhesins, in particular *sfa, papC* and *fimA*, in most of the isolates from urine and feces but the pathogenic significance of this combination remains to be clarified (Kadhum et al., 2008). A combination of more than one cyclomodulin gene *(cif-cdt; cnf-cdt)* was found. The frequent association found between *cdt* and *cnf* genes was also reported by other authors and it has been hypothesized that a synergism exists between these two cyclomodulins (Mainil et al., 2003). In isolates of bovine origin all cnf2-positive *E. coli* harbored the cdt-III gene, confirming they are co-localized on the Vir plasmid of virulence (Van Bost et al., 2003b). Isolates with a complete *cif cnf* or *cdt* gene induced CPE showing the functionality of correlated proteins, while isolates presented only the internal region of *cif* did not. In these isolates, the *cif* genes showed mutations at the 3^0-end of the gene (C-termi-nal sequence) that led to a non-functional truncated CIF protein.

Considering the results of the present study, it can be concluded that cyclomodulins are strongly correlated to pathogenic *E. coli* isolates. Moreover, *cnf*- and *cdt*-positive *E. coli* isolates harbored a great number of extraintestinal virulence factors, and belonged predominantly to phylogroups B2 and D, regardless of their origin, while cif-positive *E. coli* isolates showing a low number of virulence factors were associated mainly with group B1 and were only found in isolates causing diarrhea.

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Table 1
Sequences, predicted size of amplified products, annealing temperatures and references of the oligonucleotide primers used in PCRs.

Gene	Primer sequence (5′ to 3′)	Product size (bp)	Annealing (°C)	Reference
cnf	TTATATAGTCGTCAAGATGGA CACTAAGCTTTACAATATTGA	760	60	Van Bost et al. (2003a)
cnf1	GGCGACAAATGCAGTATTGCTTGG GACGTTGGTTGCGGTAATTTTGGG	552	63	Pass et al. (2000)
cnf2	GTGAGGCTCAACGAGATTATGCACTG CCAACGCTTCTTCTTCAGTTGTTCCTC	839	63	Pass et al. (2000)
cdtB	GAAAGTAAATGGAATATAAATGTCCG AAATCACCAAGAATCATCCAGTTA	466	55	Tóth et al. (2003)
cdtB	GAAAATAAATGGAACACACATGTCCG AAATCTCCTGCAATCATCCAGTTA	466	55	Tóth et al. (2003)
cdtB-I	CAATAGTCGCCCACAGGA ATAATCAAGAACACCACCAC	411	55	Tóth et al. (2003)
cdtB-II	GAAAGTAAATGGAATATAAATGTCCG TTTGTGTTGCCGCCGCTGGTGAAA	556	55	Tóth et al. (2003)
cdtB-III	GAAAGTAAATGGAATATAAATGTCCG TTTGTGTCGGTGCAGCAGGGAAAA	555	55	Tóth et al. (2003)
cdtB-IV	CCTGATGGTTCAGGAGGCTGGTTC TTGCTCCAGAATCTATACCT	350	55	Tóth et al. (2003)
cdtB-V	AGCACCCGCAGTATCTTTGA AGCCTCTTTTATCGTCTGGA	1363	52	Bielaszewska et al. (2004)
cif internal	AACAGATGGCAACAGACTGG AGTCAATGCTTTATGCGTCAT	383	57	Marchès et al. (2003)
cif complete	CGTGAAGGAGTGAGATATGAAAGACATTACC CTGAATCATTTTACCGTATGG	948	53	Marchès et al. (2003)
cif mut1	TGACGCATAAAGCATTG CTAACTACATAGTGATTTTATTATC	245	47	This study
cif mut2	ACATCAAACAGATGGCAACAG GAGATTCAGGGGGCCAGTCT	439	57	This study

Table 2
Detection and distribution of cyclomodulins among *E. coli* isolated from feces of calves and pups with and without diarrhea, and from urine of adult dogs with cystitis.

Sample	Animal	Origin	No. (%) of isolates cyclomodulin positive					
			Total	*cif* alone	*cnf* alone	*cdt* alone	*cif–cdt*	*cdt–cnf*
Feces ($n = 316$)	Calves	Diarrhea ($n = 255$)	32	10 (3.9)	4 (1.6)	11 (4.3)	5 (2.0)	3 (1.2)
		Without diarrhea ($n = 21$)	0	–	–	–	–	–
	Pups	Diarrhea ($n = 29$)	14	4 (13.8)	4 (13.8)	2 (7.0.8)	1 (3.4)	3 (10.3)
		Without diarrhea ($n = 11$)	1	–	1 (9.0)	–	–	–
Urine ($n = 58$)	Adult dogs	Cystitis($n = 58$)	32	–	28 (48.3)	2 (3.4)	–	2 (3.4)
Total ($n = 374$)			80	14 (3.7)	37 (9.9)	15 (4.0)	6 (1.6)	8 (2.1)

Table 3
Distribution of cyclomodulins and additional virulence genes in *E. coli* isolates.

Cyclomodulin	No. (%) of isolates												
	Total ($n = 80$)		P^a	A ($n = 9$)		B1 ($n = 12$)		P	B2 ($n = 48$)		P	D ($n = 11$)	
	Feces $n = 48$	Urine $n = 32$		Feces $n = 8$	Urine $n = 1$	Feces $n = 10$	Urine $n = 2$		Feces $n = 20$	Urine $n = 28$		Feces $n = 10$	Urine $n = 1$
cif ($n = 14$)	14 (29.2)	–	<0.01	2 (25.0)	–	9 (90.0)	–	<0.05	3 (15.0)	–		–	–
cnf ($n = 37$)	9 (18.7)	28 (87.5)	<0.01	2 (25.0)	1 (100)	–	2 (100)		6 (30.0)	25 (89.3)	<0.01	1 (10.0)	–
cdt ($n = 15$)	13 (27.1)	2 (6.2)	<0.05	2 (25.0)	–	–	–		3 (15.0)	2 (7.1)		8 (80.0)	–
cif–cdt ($n = 6$)	6 (12.5)	–		1 (12.5)	–	–	–		5 (25.0)	–		–	–
cnf–cdt ($n = 8$)	6 (12.5)	2 (6.2)		1 (12.5)	–	1 (10.0)	–		3 (15.0)	1 (3.6)		1 (10.0)	1 (100)

[a] *P* value (by Fisher exact test) are shown where *P* is <0.05.

Table 4
Distribution of cyclomodulins among *E. coli* isolates according to the four phylogenetic groups.

Cyclomodulin	No. (%) of isolates												
	Total ($n = 80$)		P[a]	A ($n = 9$)		B1 ($n = 12$)		P	B2 ($n = 48$)		P	D ($n = 11$)	
	Feces $n = 48$	Urine $n = 32$		Feces $n = 8$	Urine $n = 1$	Feces $n = 10$	Urine $n = 2$		Feces $n = 20$	Urine $n = 28$		Feces $n = 10$	Urine $n = 1$
cif ($n = 14$)	14 (29.2)	–	<0.01	2 (25.0)	–	9 (90.0)	–	<0.05	3 (15.0)	–		–	–
cnf ($n = 37$)	9 (18.7)	28 (87.5)	<0.01	2 (25.0)	1 (100)	–	2 (100)		6 (30.0)	25 (89.3)	<0.01	1 (10.0)	–
cdt ($n = 15$)	13 (27.1)	2 (6.2)	<0.05	2 (25.0)	–	–	–		3 (15.0)	2 (7.1)		8 (80.0)	–
cif–cdt ($n = 6$)	6 (12.5)	–		1 (12.5)	–	–	–		5 (25.0)	–		–	–
cnf–cdt ($n = 8$)	6 (12.5)	2 (6.2)		1 (12.5)	–	1 (10.0)	–		3 (15.0)	1 (3.6)		1 (10.0)	1 (100)

[a] P value (by Fisher exact test) are shown where P is <0.05.