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# Characterization of enterotoxigenic *E. coli* (ETEC), Shiga-toxin producing *E. coli* (STEC) and necrotoxigenic *E. coli* (NTEC) isolated from diarrhoeic Mediterranean water buffalo calves (*Bubalus bubalis*)

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ABSTRACT

Two hundred and twenty *Escherichia coli* isolates from 314 Mediterranean water buffalo calves less than 4 weeks old affected by severe diarrhoea with a lethal outcome were characterized for the presence of the virulence factors LT, ST, Stx1, Stx2, haemolysins, intimin, CNF1, CNF2, CDT-I, CDT-II, CDT-III, CDT-IV, and F17-related fimbriae (F17a, F17b, F17c, F17d). The prevalence of ETEC, STEC and NTEC were 1.8%, 6.8% and 20.9%, respectively. The ETEC isolates were all LT-positive and ST-negative. The STEC isolates were all Stx and intimin-positive, with Stx1 (80%) more frequent than Stx2 (27%). The NTEC isolates were all CNF and Hly-positive, with CNF2 (83%) more frequent than CNF1 (22%). Susceptibility assays to 11 antimicrobials displayed high rates of resistance (>30%) to antimicrobials tested. These data show that the most prevalent strains in diarrhoeic water buffalo calves were NTEC, mostly CNF2 and HlyA-positive, with strong associations CNF2/CDT-III and CNF2/F17c.



*Escherichia coli* infections can be caused both in humans and animals by different pathovars, most frequently identified as enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC) including Shiga-toxin producing *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC). These pathovars can induce a variety of diseases, such as diarrhoea, haemorrhagic colitis, and extra intestinal infections (Croxen and Finlay, 2010). Their pathogenic mechanisms can be attributed to different virulence factors including enterotoxins and colonization factors such as flagella, fimbriae, capsule, lipopolysaccharide and adhesins (Croxen and Finlay, 2010; Gyles and Fairbrother, 2010). In addition other pathovars such as necrotoxigenic *E. coli* (NTEC) can often be isolated from extra intestinal infections, and are found to possess two cytotoxic necrotizing factors (CNF1 and CNF2), as well as a cytolethal distending toxin (CDT). Some or all of these pathovars can also express other virulence factors such as intimin, encoded by the *eae* gene (Croxen and Finlay, 2010), and haemolysins, acting as pore-forming cytolysins on eukaryotic target cells (Mainil and Daube, 2005).

The Mediterranean water buffalo (*Bubalus bubalis*) is one of the most important livestock species bred in Italy. Its economic importance is due to the production of the worldwide famous mozzarella cheese (*Mozzarella di Bufala Campana*). High mortality rates in water buffalo calves less than 4 weeks old are caused by gastroenteric pathologies primarily characterized by diarrhoea. The main etiological agents are *E. coli*, *Salmonella* spp., *Clostridium perfringens*, rotavirus, coronavirus, and *Cryptosporidium* spp. (Fagiolo et al., 2005). Among these pathogens, a predominant role is played by *E. coli*, either alone or in combination with other microorganisms (Fagiolo et al., 2005).

ETEC and STEC have been shown to be primarily affecting water buffalo calves, and the Mediterranean water buffalo is recognized to be an important reservoir of *E. coli* O157 (Galiero et al., 2005). A significant correlation has also been found between the presence of STEC and diarrhoea episodes in calves bred in Vietnam and Bangladesh (Islam et al., 2008; Vu-Khac and Cornick, 2008).

The spread of such pathogens among domestic ruminant herds might cause additional concern related to the emergence and dissemination of antibiotic resistant bacteria in response to the wide use of antimicrobial molecules to address infectious diseases in young animals (van den Bogaard and Stobberingh, 2000). The aims of this study were to investigate the presence of ETEC, STEC and NTEC in water buffalo calves affected by diarrhoeic syndrome and to characterize the isolates for the presence of virulence factors and antibiotic resistance.

Intestinal contents from 314 water buffalo calves less than 4 weeks old were collected from 32 different farms located in the Campania region within a range of about 50 km during the years

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2006–2009. The animals were all female, bred in single cages and fed with powdered milk. The sampled animals were affected by diarrhoeic syndrome and were sent to the Istituto Zooprofilattico Sperimentale del Mezzogiorno for post-mortem analysis. Pools of fecal material from small and large intestines were aseptically collected, kept at 4 °C and, within 8 h, were processed for the detection of *E. coli*. Fecal samples (10 g) were homogenized and serially diluted in saline peptone water, plated onto MacConkey's agar (Oxoid, Hampshire, UK) and incubated overnight at 37 °C. *E. coli* was considered as plausibly related to the diarrhoeic syndrome only when exhibiting a concentration of at least $10^8$ CFU/g of intestinal content (Acres, 1985), and the corresponding samples were therefore considered as positive to *E. coli* detection. Three colonies from all the *E. coli*-positive samples were chosen and confirmed by standard biochemical tests performed with the Vitek 2 compact instrument (BioMérieux, Craponne, France).

All the *E. coli* isolates were screened by PCR for the presence of the genes encoding for the virulence factors listed in Table 1. Bacterial DNA was extracted by boiling a single colony suspended in 100 μl of water at 100 °C for 10 min and pelleting cellular debris. DNA amplification was performed in a final reaction volume of 50 μl containing 10 μl of template DNA, reaction buffer 1×, $MgCl_2$ 1.2 mM, dNTPs 0.2 mM each, 50 pmol of each primer, 2 U of Taq polymerase (Roche Diagnostics, Basel, Switzerland). Primers and PCR conditions for each amplification are summarized in Table 1. PCR products were resolved by electrophoresis on 2% agarose gels and visualized under UV light after ethidium bromide staining.

Heat-stable enterotoxins (STs) were detected by the competitive immunoenzymatic assay *E. coli* ST EIA (Oxoid, Hampshire, UK) as described by the manufacturer. Heat-labile enterotoxin (LT), Shiga-toxins (Stx1 and Stx2) and cytotoxic necrotizing factors (CNF1 and CNF2) production was tested by a cytotoxicity assay on Vero cells as previously described (Caprioli et al.,1983). Cell monolayers were incubated at 37 °C with 5% $CO_2$ and examined after 24, 48, and 72 h by a phase contrast inverted microscope (Zeiss, Göttingen, Deutschland). LT and Stxs activities were determined by morphological changes in the exposed cells according to Konowalchuk et al. (1977). All the tests performed on Vero cells included the use of proper positive and negative controls (Table 1). Haemolysin production was evaluated based on the method by Beutin et al. (1989) by inoculation of bacterial strains onto blood agar base (Difco Laboratories, Detroit, MI) supplemented with 10 mM $CaCl_2$ and sheep blood cells (Oxoid) washed with PBS. The plates were incubated at 37 °C for 24 h and observed for haemolysis after 3 h (for expression of α-haemolysin, hlyA) and 24 h (for enterohaemolysin, Ehly). ETEC isolates grown on Minca agar

**Table 1**
PCR primers and conditions used in this study.

| Primer | Sequence (5′–3′) | Target gene | PCR product | Reference strains | Reference | PCR Conditions | |
|---|---|---|---|---|---|---|---|
| Stx1F<br>Stx1R | CAGTTAATGTGGTGGCGAAGG<br>CACCAGACAATGTAACCGCTG | *stx*1 | 348 bp | C2103;<br>ED 669 | Vidal et al. (2005) | 35× | 90 s at 94 °C<br>90 s at 60 °C<br>90 s at 72 °C |
| Stx2F<br>Stx2R | ATCCTATTCCCGGGAGTTTACG<br>GCGTCATCGTATACACAGGAGC | *stx2* | 584 bp | C210–03 | Vidal et al. (2005) | 35× | 90 s at 94 °C<br>90 s at 60 °C<br>90 s at 72 °C |
| EaeF<br>EaeR | TCAATGCAGTTCCGTTATCAGTT<br>GTAAAGTCCGTTACCCCAACCTG | *eae* | 482 bp | C210–03;<br>ED 669 | Vidal et al. (2005) | 35× | 90 s at 94 °C<br>90 s at 60 °C<br>90 s at 72 °C |
| LtF<br>LtR | GCACACGGAGCTCCTCAGTC<br>TCCTTCATCCTTTCAATGGCTTT | *ltII* | 218 bp | H10407 | Vidal et al. (2005) | 35× | 90 s at 94 °C<br>90 s at 60 °C<br>90 s at 72 °C |
| StF<br>StR | AAAGGAGAGCTTCGTCACATTTT<br>AATGTCCGTCTTGCGTTAGGAC | *stII* | 129 bp | EA-11 | Vidal et al. (2005) | 35× | 90 s at 94 °C<br>90 s at 60 °C<br>90 s at 72 °C |
| Cnf1F<br>Cnf1R | GGGGGAAGTACAGAAGAATTA<br>TTGCCGTCCACTCTCTCACCAGT | *cnf1* | 1111 bp | EF-176 | Toth et al. (2003) | 30× | 60 s at 94 °C<br>60 s at 55 °C<br>60 s at 72 °C |
| Cnf2F<br>Cnf2R | TATCATACGGCAGGAGGAAGCACC<br>GTCACAATAGACAATAATTTTCCG | *cnf2* | 1240 bp | EF-147 | Toth et al. (2003) | 30× | 60 s at 94 °C<br>60 s at 55 °C<br>60 s at 72 °C |
| Cdt1F<br>Cdt1R | CAATAGTCGCCCACAGGA<br>ATAATCAAGAACACCACCAC | *cdt-I* | 411 bp | EF-133 | Toth et al. (2003) | 30× | 60 s at 94 °C<br>60 s at 55 °C<br>60 s at 72 °C |
| Cdt2F<br>Cdt2R | GAAAATAAATGGAATATAAATGTCCG<br>TTTGTGTTGCCGCCGCTGGTGAAA | *cdt-II* | 556 bp | 9142–88 | Toth et al. (2003) | 30× | 60 s at 94 °C<br>60 s at 55 °C<br>60 s at 72 °C |
| Cdt3F<br>Cdt3R | GAAAATAAATGGAATATAAATGTCCG<br>TTTGTGTCGGTGCAGCAGGGAAAA | *cdt-III* | 555 bp | EF-147 | Toth et al. (2003) | 30× | 60 s at 94 °C<br>60 s at 55 °C<br>60 s at 72 °C |
| Cdt4F<br>Cdt4R | CCTGATGGTTCAGGAGGCTGGTTC<br>TTGCTCCAGAATCTATACCT | *cdt-IV* | 350 bp | E253 | Toth et al. (2003) | 30× | 60 s at 94 °C<br>60 s at 55 °C<br>60 s at 72 °C |
| F17aF<br>F17aR | GCTGGAAGGGTGCAATACGCCTG<br>ATTCGTAACCCGCTCTCGTCC | *f17a* | 321 bp | 25KH9 | Bertin et al. (1996) | 25× | 120 s at 94 °C<br>60 s at 55 °C<br>60 s at 72 °C |
| F17bF<br>F17bR | CAACTAACGGGATGTACAGTTTC<br>ATTCGTAACCCGCTCTCGTCC | *f17b* | 323 bp | S5 | Bertin et al. (1996) | 25× | 120 s at 94 °C<br>60 s at 55 °C<br>60 s at 72 °C |
| F17cF<br>F17cR | GCAGGAACCGCTCCCTTGGC<br>CAACTAACGGGATGTACAGTTTC | *f17c* | 416 bp | 31A | Bertin et al. (1996) | 25× | 120 s at 94 °C<br>60 s at 55 °C<br>60 s at 72 °C |
| F17dF<br>F17dR | GATAGTCATAACCTTAATATTGCA<br>CAACTAACGGGATGTACAGTTTC | *f17d* | 239 bp | 111KH86 | Bertin et al. (1996) | 25× | 120 s at 94 °C<br>60 s at 55 °C<br>60 s at 72 °C |

(Guinée et al., 1977) plus IsoVitalex (Becton Dickinson, Heidelberg, Germany) were tested for the presence of the fimbrial antigen F5 (K99) by latex agglutination test with a specific monoclonal antibody (FIMBREX K99 kit, VLA, Weybridge, UK).

STEC isolates were characterized for the O-serogroups O26, O103, O111, O145 and O157 by Real-Time PCR as previously described by Perelle et al. (2004, 2005).

Antimicrobial susceptibilities were determined by the agar disk diffusion method on Mueller–Hinton Agar (Oxoid) according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). All the *E. coli* isolates were assayed for susceptibility to the antimicrobials listed in Table 2. Inhibition diameters were measured and interpreted as resistant, intermediate or susceptible according to CLSI guidelines (CLSI, 2008).

The prevalence of *E. coli* responsible for gastroenteritis in Mediterranean water buffalo calves is poorly investigated. However, epidemiologic studies of both bovine and water buffalo calves have implicated *E. coli* as the major cause of neonatal diarrhoea (Fagiolo et al., 2005; Foster and Smith, 2009). Our study shows a prevalence of about 70% (220 out of 314) of *E. coli* in water buffalo calves less than 4 weeks old affected by diarrhoeic syndrome with a lethal outcome. The collected isolates were tested by phenotypic and molecular analyses for the identification of ETEC, STEC and NTEC. As a result of the molecular screening for virulence factors, four ETEC (1.8%), 15 STEC (6.8%) and 46 NTEC (20.9%) were identified. The results of the phenotypic assays were consistent with those obtained by PCR, except for a few cases. In fact, two false negative STEC and three NTEC were observed by phenotypic tests. A considerable number (155) of isolates did not harbour the virulence factors investigated and were most probably part of the gut's natural commensal microflora.

All the ETEC isolates could produce LT, but were negative to ST detection (Table 3). Unlike bovine calves, our study shows that in diarrhoeic water buffalo calves ETEC strains are infrequent. The differences between bovine and water buffalo ETEC strains also lie in the expressed virulence factors. Indeed, water buffalo ETEC strains exhibited the production of LT toxin, while ETEC strains of bovine origin have been shown to primarily produce ST toxin (Holland, 1990), even if ETEC with the genes for LT have been isolated also from cows, buffaloes and mithuns (Gyles and Fairbrother, 2010; Rajkhowa et al., 2009). Moreover, our results show that, unlike ETEC strains from diarrhoeic bovine calves (Gyles and Fairbrother, 2010), ETEC isolates from diarrhoeic water buffalo calves do not possess the F5 antigen. As a practical consequence, the common use of scours vaccines containing *E. coli* expressing the F5 fimbria is unsuitable to prevent ETEC infections in Mediterranean water buffalo calves.

The STEC isolates were all Stx and intimin-positive, and were therefore classified as attaching and effacing *E. coli,* AEEC (Gyles and Fairbrother, 2010). In particular 11 isolates were Stx1-positive, three isolates were Stx2-positive, and one isolate was positive to both Stx1 and Stx2 (Table 3). Among these isolates, six also exhibited the production of enterohaemolysin (Ehly) and were therefore classified as EHEC (Table 3). Leomil et al. (2003)) reported a frequency of STEC in bovine diarrhoeic and non-diarrhoiec calves of 12.7%, among which the incidence of *eae* and Ehly-positive STEC was 18.2%. A recent study on buffaloes at slaughterhouse in Bangladesh reported a prevalence of STEC of 37.9%, mostly *eae*-positive, with an incidence of 14.4% of the serotype O157 (Islam et al., 2008). In Vietnam, a similar study showed that intimin-positive STEC strains could be recovered from 27% of rectal swabs from randomly selected buffaloes, but no serotype O157 could be isolated (Vu-Khac and Cornick, 2008). In Brazil Oliveira et al. (2007) described healthy water buffalo as an important reservoir of STEC, while in Italy adult water buffalo has been reported as a natural reservoir of the serotype O157 (Galiero et al., 2005). In the present study, *eae*-positive STEC were recovered from 6.8% of the *E. coli* isolates but none of the serotypes O26, O103, O111, O145 or O157 was identified. The difference between our results and the previous evidence of a prevalence of the serotype O157 in water buffaloes might reflect a different distribution of *E. coli* serotypes between young and adult animals. Instead, the prevalence of *eae*-positive STEC mostly Stx1-positive observed in this study in water buffalo calves, is consistent with data reported for diarrhoeic bovine calves where Stx1 is frequently associated with *eae*-positive strains and the *eae* gene is more frequently found in STEC from calves compared to STEC from adult cattle (Mainil et al., 1993; Sandhu et al., 1996).

NTEC was the most prevalent pathovar (20.9%) among diarrhoeic water buffalo calves. All the NTEC strains could produce CNF; in particular 36 isolates were CNF2-positive, eight were CNF1-positive and two isolates were positive to both CNF1 and CNF2 (Table 3). Among CNF2-positive NTEC, 35 isolates had the

**Table 3**
Distribution of virulence factors in *Escherichia coli* isolates collected from diarrhoeic water buffalo calves less than 4 weeks old.

| *E. coli* type | Frequency (%) | Virulence factors[a] | No. of isolates |
|---|---|---|---|
| ETEC | 1.8 | LT | 4 |
| | | ST | 0 |
| AEEC | 4.1 | Stx1; *eae* | 5 |
| | | Stx2; *eae* | 3 |
| | | Stx1; Stx2; *eae* | 1 |
| EHEC | 2.7 | Stx1; *eae*; Ehly | 6 |
| NTEC | 20.9 | CNF1; HlyA | 8 |
| | | CNF1; CNF2; CDT-III; HlyA | 2 |
| | | CNF2; HlyA | 2 |
| | | CNF2; F17c; HlyA | 1 |
| | | CNF2; CDT-III; HlyA | 28 |
| | | CNF2; CDT-III; F17c; HlyA | 5 |
| *E. coli* | 70.5 | None detected | 155 |
| Total | | | 220 |

[a] Virulence factors included in the study: heat-labile enterotoxin (LT), heat-stable enterotoxins (ST) Shiga-toxins (Stx1 and Stx2), α-haemolysin (HlyA), enterohaemolysin (Ehly), intimin (*eae*), cytotoxic necrotizing factors (CNF1 and CNF2), cytolethal distending toxins (CDT-I, CDT-II, CDT-III and CDT-IV), and F17 fimbriae family (F17a, F17b, F17c and F17d).

**Table 2**
Antimicrobial susceptibility patterns of 220 *Escherichia coli* isolates collected from 314 diarrhoeic water buffalo calves less than 4 weeks old.

| *E. coli* (%)[b] | Antimicrobials[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amp | Ot | Sxt | Ct | N | Na | Apr | Cn | Ub | Enr | Amc |
| *R* | 81.8 | 74 | 45.9 | 41.5 | 48.5 | 49.3 | 33.5 | 31.2 | 31.9 | 30.6 | 42.6 |
| *I* | 9.5 | 8 | 5.3 | 12.2 | 29.1 | 12.8 | 19.3 | 9.6 | 11.1 | 11 | 19 |
| *S* | 8.7 | 18 | 48.7 | 46.3 | 22.2 | 37.7 | 47.1 | 59.1 | 57.2 | 58.3 | 38.3 |

[a] Antimicrobial molecules included in the study: ampicillin (Amp) – 10 μg, oxytetracycline (Ot) – 30 μg, sulphamethoxazole/trimethoprim (Sxt) – 25 μg, colistin (Ct) – 10 μg, neomycin (N) – 30 μg, nalidixic acid (Na) – 30 μg, apramycin (Apr) – 15 μg, gentamicin (Cn) – 10 μg, flumequine (Ub) – 30 μg, enrofloxacin (Enr) – 5 μg and amoxicillin/clavulanic acid (Amc) – 30 μg.
[b] Percentage of resistant (*R*), intermediate susceptible (*I*) and susceptible (*S*) *E. coli* isolates.

*cdt*-III gene, and six isolates produced the F17c fimbria, one of the F17-related fimbriae (Table 3). CNF-producing *E. coli* have already been detected in association with both diarrhoeic and healthy bovine calves (Blanco et al., 1993; Burns et al., 1996; Orden et al., 1999, 2002; Van Bost et al., 2001), and our report shows high similarities between NTEC from bovine and water buffalo species. In fact, water buffalo NTEC frequency appeared comparable to those exhibited by NTEC recovered from both diarrhoeic (ranging from 8% to 23.3%) and healthy bovine calves (from 9.9% to 35.3%). All the collected water buffalo NTEC isolates also exhibited the production of α-haemolysin (HlyA), as elsewhere described for most NTEC of animal and human origin (Caprioli et al., 1989). Moreover, as for bovine NTEC, most NTEC from diarrhoeic water buffalo calves were CNF2-positive, and exhibited a strong association between the virulence factors CNF2 and F17 (Mainil et al., 1999; Orden et al., 1999; Van Bost et al., 2001), in this case F17c. Water buffalo NTEC also showed a strong association between CNF2 and CDT-III. The large presence of NTEC in diarrhoeic water buffalo calves, and the number of expressed virulence factors, highlight the pathogenic potential of this pathovar, which is stronger considering the possibility of exchanges between water buffalo and cattle. In fact, although most farms in the Campania region breed single species, either bovine or water buffalo, and animals are not grazed, there is still a high chance of contagion as many farms still lack biosecurity requirements necessary to control the entry and the spread of diseases on the herd.

All the *E. coli* isolates were tested for susceptibility to 11 antimicrobials among those most commonly used in veterinary medicine. They were all characterized by multi-drug resistance profiles, exhibiting resistance to at least four unrelated molecules variably combined, and nine isolates were found to be resistant to all the antimicrobials included in the study. High rates of resistance were observed for Amp (81.8%) and Ot (74%), while the lowest resistance rates were exhibited for Ub, Cn and Enr with resistance percentages of 31.9%, 31.2% and 30.6%, respectively (Table 2). Multi-drug resistant *E. coli* have been isolated from many different species, including bovine, pigs and sheep (Enne et al., 2008; Lee, 2009). Resistance rates exhibited by the *E. coli* strains isolated from Mediterranean water buffalo calves included in this study appear alarmingly high, above all those observed for the newer molecules. The use of quinolones and fluoroquinolones in human medicine urged the European Commission to start a referral procedure for all veterinary medical products containing these classes of antimicrobials, aiming to promote their careful use in veterinary treatments (Directive 2001/82/EC; SANCO/6876/2009r6). Prophylaxis is essential to prevent the occurrence of infectious diseases; in general, the upgraded health and welfare status, and the availability of specific vaccines, especially autogenous bacterins (custom bacterins), could result in a reduction of the use of antibiotics, and might, consequently, limit the emergence of antimicrobial resistances.

In conclusion, the results show that the most prevalent strains in diarrhoeic water buffalo calves were NTEC followed by *eae*-positive STEC and ETEC. The virulence factors associated with the NTEC strains were mostly CNF2 and haemolysin, with CNF2 exhibiting a strong association with CDT-III and with F17c. These results might therefore be useful for the development of effective prophylaxis and therapy protocols for the control of *E. coli* infections in water buffalo farms.

## Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the article.

## References


Acres, S.D., 1985. Enterotoxigenic *Escherichia coli* infections in newborn calves: a review. Journal of Dairy Science 68, 229–256.

Bertin, Y., Martin, C., Oswald, E., Girardeau, J.P., 1996. Rapid and specific detection of F17-related pilin and adhesin genes in diarrheic and septicemic *Escherichia coli* strains by multiplex PCR. Journal of Clinical Microbiology 34, 2921–2928.

Beutin, L., Montenegro, M.A., Orskov, I., Orskov, F., Prada, J., Zimmermann, S., Stephan, R., 1989. Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. Journal of Clinical Microbiology 27, 2559–2564.

Blanco, M., Blanco, J., Blanco, J.E., Ramos, J., 1993. Enterotoxigenic, verotoxigenic, and necrotoxigenic *Escherichia coli* isolated from cattle in Spain. American Journal of Veterinary Research 54, 1446–1451.

Burns, A.L., Ball, H.J., Finlay, D.A., 1996. CNF producing *Escherichia coli* isolated from cattle in Northern Ireland. Veterinary Microbiology 49, 235–241.

Caprioli, A., Falbo, V., Roda, L.G., Ruggeri, F.M., Zona, C., 1983. Partial purification and characterization of an *Escherichia coli* toxic factor that induces morphological cell alterations. Infection and Immunity 39, 1300–1306.

Caprioli, A., Falbo, V., Ruggeri, F.M., Minelli, F., Orskov, I., Donelli, G., 1989. Relationship between cytotoxic necrotizing factor production and serotype in haemolytic *Escherichia coli*. Journal of Clinical Microbiology 27, 758–761.

CLSI, 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, third ed. Approved Standard. CLSI document M31-A3. Clinical and Laboratory Standards Institute, Wayne, PA.

Croxen, M.A., Finlay, B.B., 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. Nature Reviews Microbiology 8, 26–38.

Directive 2001/82/EC (OJ L311, 28.11.2001 p. 1).

Enne, V.I., Cassar, C., Sprigings, K., Woodward, M.J., Bennett, P.M., 2008. A high prevalence of antimicrobial resistant *Escherichia coli* isolated from pigs and a low prevalence of antimicrobial resistant *E. Coli* from cattle and sheep in Great Britain at slaughter. FEMS Microbiology Letters 278, 193–199.

Fagiolo, A., Roncoroni, C., Lai, O., Borghese, A., 2005. Buffalo Pathologies. In: Borghese, A. (Ed.), Buffalo Production and Research. FAO Regional Office for Europe Inter-Regional Cooperative Research Network on Buffalo, Rome, pp. 249–296.

Foster, D.M., Smith, G.W., 2009. Pathophysiology of diarrhea in calves. Veterinary Clinics of North America: Food Animal Practice 25, 13–36.

Galiero, G., Conedera, G., Alfano, D., Caprioli, A., 2005. Isolation of verocytotoxin-producing *Escherichia coli* O157 from water buffaloes (*Bubalus bubalis*) in Southern Italy. Veterinary Record 156, 382–383.

Guinée, P.A., Veldkamp, J., Jansen, W.H., 1977. Improved minca medium for the detection of K99 antigen in calf enterotoxigenic strains of *Escherichia coli*. Infection and Immunity 15, 676–678.

Gyles, C.L., Fairbrother, J.M., 2010. *Escherichia coli*. In: Gyles, C.L., Prescott, J.F., Thoen, C.O. (Eds.), Pathogenesis of Bacterial Infections in Animals. Blackwell Publishing, Ames, pp. 267–308.

Holland, R.E., 1990. Some infectious causes of diarrhea in young farm animals. Clinical Microbiology Reviews 3, 345–375.

Islam, M.A., Mondol, A.S., de Boer, E., Beumer, R.R., Zwietering, M.H., Talukder, K.A., Heuvelink, A.E., 2008. Prevalence and genetic characterization of shiga toxin-producing *Escherichia coli* isolates from slaughtered animals in Bangladesh. Applied and Environmental Microbiology 74, 5414–5421.

Konowalchuk, J., Speirs, J.I., Stavric, S., 1977. Vero response to a cytotoxin of *Escherichia coli*. Infection and Immunity 18, 775–779.

Lee, J.H., 2009. Antimicrobial resistance of *Escherichia coli* O26 and O111 isolates from cattle and their characteristics. Veterinary Microbiology 135, 401–405.

Leomil, L., Aidar-Ugrinovich, L., Guth, B.E., Irino, K., Vettorato, M.P., Onuma, D.L., de Castro, A.F., 2003. Frequency of Shiga toxin-producing *Escherichia coli* (STEC) isolates among diarrheic and non-diarrheic calves in Brazil. Veterinary Microbiology 97, 103–109.

Mainil, J.G., Jacquemin, E.R., Kaeckenbeeck, A.E., Pohl, P.H., 1993. Association between the effacing (*eae*) gene and the Shiga-like toxin-encoding genes in *Escherichia coli* isolates from cattle. American Journal of Veterinary Research 54, 1064–1068.

Mainil, J.G., Jacquemin, E., Pohl, P., Fairbrother, J.M., Ansuini, A., Le Bouguénec, C., Ball, H.J., De Rycke, J., Oswald, E., 1999. Comparison of necrotoxigenic *Escherichia coli* isolates from farm animals and from humans. Veterinary Microbiology 70, 123–135.

Mainil, J.G., Daube, G., 2005. Verotoxigenic *Escherichia coli* from animals, humans and foods: who's who? Journal of Applied Microbiology 98, 1332–1344.

Oliveira, M.G., Brito, J.R., Carvalho, R.R., Guth, B.E., Gomes, T.A., Vieira, M.A., Kato, M.A., Ramos, I.I., Vaz, T.M., Irino, K., 2007. Water buffaloes (*Bubalus bubalis*) identified as an important reservoir of Shiga toxin-producing *Escherichia coli* in Brazil. Applied and Environmental Microbiology 73, 5945–5948.

Orden, J.A., Ruiz-Santa-Quiteria, J.A., Cid, D., García, S., de la Fuente, R., 1999. Prevalence and characteristics of necrotoxigenic *Escherichia coli* (NTEC) strains isolated from diarrhoeic dairy calves. Veterinary Microbiology 66, 265–273.

Orden, J.A., Cid, D., Ruiz-Santa-Quiteria, J.A., García, S., Martínez, S., de la Fuente, R., 2002. Verotoxin-producing *Escherichia coli* (VTEC), enteropathogenic *E Coli* (EPEC) and necrotoxigenic *E. coli* (NTEC) isolated from healthy cattle in Spain. Journal of Applied Microbiology 93, 29–35.

Perelle, S., Dilasser, F., Grout, J., Fach, P., 2004. Detection by 5'-nuclease PCR of Shiga-toxin producing *Escherichia coli* O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world's most frequent clinical cases. Molecular and Cellular Probes 18, 185–192.

Perelle, S., Dilasser, F., Grout, J., Fach, P., 2005. Detection of *Escherichia coli* serogroup O103 by real-time polymerase chain reaction. Journal of Applied Microbiology 98, 1162–1168.

Rajkhowa, S., Hussain, I., Rajkhowa, C., 2009. Detection of heat-stable and heat-labile enterotoxin genes of *Escherichia coli* in diarrhoeic faecal samples of mithun (*Bos frontalis*) calves by polymerase chain reaction. Journal of Applied Microbiology 106, 455–458.

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Sandhu, K.S., Clarke, R.C., McFadden, K., Brouwer, A., Louie, M., Wilson, J., Lior, H., Gyles, C.L., 1996. Prevalence of the *eaeA* gene in verotoxigenic *Escherichia coli* strains from dairy cattle in Southwest Ontario. Epidemiology and Infection 116, 1–7.

Toth, I., Hérault, F., Beutin, L., Oswald, E., 2003. Production of cytolethal distending toxins by pathogenic *Escherichia coli* strains isolated from human and animal sources: establishment of the existence of a new *cdt* variant (type IV). Journal of Clinical Microbiology 41, 4285–4291.

Van Bost, S., Bâbe, M.H., Jacquemin, E., Mainil, J., 2001. Characteristics of necrotoxigenic *Escherichia coli* isolated from septicemic and diarrheic calves between 1958 and 1970. Veterinary Microbiology 82, 311–320.

van den Bogaard, A.E., Stobberingh, E.E., 2000. Epidemiology of resistance to antibiotics Links between animals and humans. International Journal of Antimicrobial Agents 14, 327–335.

Vidal, M., Kruger, E., Durán, C., Lagos, R., Levine, M., Prado, V., Toro, C., Vidal, R., 2005. Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. Journal of Clinic Microbiology 43, 5362–5365.

Vu-Khac, H., Cornick, N.A., 2008. Prevalence and genetic profiles of Shiga toxin-producing *Escherichia coli* strains isolated from buffaloes, cattle, and goats in central Vietnam. Veterinary Microbiology 126, 356–363.