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

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