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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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- Antimicrobial resistance

**A B S T R A C T**

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.

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DNA amplification was performed in a final reaction volume of 25 μl containing 10 mM Tris–HCl, 150 mM KCl, 0.01% Tween 20, 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 were 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 ElA (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% CO2 and examined after 24, 48, and 72 h by a phase contrast inverted microscope (Zeiss, Göttingen, Deutschland). LT and STxxs 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 CaCl2 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, Ehy). ETEC isolates grown on Minca agar

### Table 1

| Primer | Sequence (5'-3') | Target gene | PCR product | Reference strains | Reference PCR Conditions |
|--------|-----------------|-------------|-------------|-------------------|--------------------------|
| Stx1F  | CAGTAAATGTGTTGCGGAAAGG | stx1        | 348 bp      | C2103; ED 669     | 35× 90 s at 94 °C; 90 s at 60 °C; 90 s at 72 °C |
| Stx1R  | CACGCACAAGTGTAAAGCTTG |            |             |                   |                          |
| Stx2F  | ATCCATATTCCCCGAGTATACCCGACC | stx2      | 584 bp      | C210–03           | 35× 90 s at 94 °C; 90 s at 60 °C; 90 s at 72 °C |
| Stx2R  | GGTGTACAGTATAACAGGGAGC |            |             |                   |                          |
| EaeF   | TCAAATGCCTCGTTGATCTGTT | eae         | 482 bp      | C210–03; ED 669   | 35× 90 s at 94 °C; 90 s at 60 °C; 90 s at 72 °C |
| EaeR   | GTAAAGTCTCAGTGATCTGTT |            |             |                   |                          |
| LfF    | GCACAGGGAGCTCTGTCATTT | stl         | 129 bp      | EA-11             | 35× 90 s at 94 °C; 90 s at 60 °C; 90 s at 72 °C |
| LfR    | TCTTTCATCCTTTCAATGCGTT |            |             |                   |                          |
| Cin1F  | GGGGGAAGTACAGAAGAATTAA | cnf1        | 1111 bp     | EF-176            | 30× 60 s at 94 °C; 60 s at 55 °C; 60 s at 72 °C |
| Cin1R  | TGGTCCTGACTCCTTCACAGTT |            |             |                   |                          |
| Cin2F  | TATCATACGAGGAGGACAACCG | cnf2        | 1240 bp     | EF-147            | 30× 60 s at 94 °C; 60 s at 55 °C; 60 s at 72 °C |
| Cin2R  | GTACAAGAAAGAATAATTTTCCC |            |             |                   |                          |
| Cdt1F  | CAATAGTCGCCACAGGA | cdt-1       | 411 bp      | EF-133            | 30× 60 s at 94 °C; 60 s at 55 °C; 60 s at 72 °C |
| Cdt1R  | ATATATCAAGAAGAACCAC |            |             |                   |                          |
| Cdt2F  | GAAAATATGGGAAATATGTCGCG | cdt-2     | 556 bp      | 9142–88           | 30× 60 s at 94 °C; 60 s at 55 °C; 60 s at 72 °C |
| Cdt2R  | TTTTGTTGCGCCGGTGGTAAGA |            |             |                   |                          |
| Cdt3F  | GAAAATATGGGAAATATGTCGCG | cdt-3     | 555 bp      | EF-147            | 30× 60 s at 94 °C; 60 s at 55 °C; 60 s at 72 °C |
| Cdt3R  | TTTTGTTGCGCCGGTGGTAAGA |            |             |                   |                          |
| Cdt4F  | CCTGATGGTCAGGGAGGTCGCC | cdt-4      | 350 bp      | E253              | 30× 60 s at 94 °C; 60 s at 55 °C; 60 s at 72 °C |
| Cdt4R  | TTGCTCAGAAATATATACCT |            |             |                   |                          |
| F17aF  | GTGAGGCGGGGGATACATTACCGCTG | f17a     | 321 bp      | 251KH             | 25× 120 s at 94 °C; 60 s at 55 °C; 60 s at 72 °C |
| F17aR  | ATCCGCTACCGCTTCTGACCT |            |             |                   |                          |
| F17bF  | CAATACGGGAGTACAGTTCGCTTC | f17b     | 323 bp      | S5                | 25× 120 s at 94 °C; 60 s at 55 °C; 60 s at 72 °C |
| F17bR  | ATTCGCTACCGCTTCTGACCT |            |             |                   |                          |
| F17cF  | GGCAAGGAGGGCTCTTGGGC | f17c       | 416 bp      | 31A               | 25× 120 s at 94 °C; 60 s at 55 °C; 60 s at 72 °C |
| F17cR  | CAATACGGGAGTACAGTTCGCTTC |            |             |                   |                          |
| F17dF  | GATAGCTGAACTTTAAATTGGC | f17d       | 239 bp      | 111KH86           | 25× 120 s at 94 °C; 60 s at 55 °C; 60 s at 72 °C |
| F17dR  | CAATACGGGAGTACAGTTCGCTTC |            |             |                   |                          |
therefore classified as attaching and effacing enterotoxigenic Escherichia coli (ETEC) expressing the F5 fimbria.

Stx1 and Stx2 were detected in 95.7% of STEC isolates. Of these, 15 isolates were Stx1-positive, 35 isolates had the Stx1 gene, and two isolates were positive to both Stx1 and Stx2. In particular, 36 isolates were CNF2-positive, eight were CNF2-positive and two isolates were positive to both CNF1 and CNF2. CNF; in particular 36 isolates were CNF2-positive, eight were CNF2-positive and two isolates were positive to both CNF1 and CNF2.

The STEC isolates were more frequently found in diarrhoeic water buffalo calves (20.9%) among diarrhoeic calves than in non-diarrhoeic calves (12.7%), among which the incidence of Stx1 and Stx2-positive STEC was 18.2%. A recent study on buffaloes at slaughterhouse in Bangladesh reported a prevalence of STEC of 37.5%, 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 Brazil Oliveira et al. (2007) described healthy water buffalo as an important reservoir of STEC. 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 CNF2 gene, and two isolates were positive to both CNF1 and CNF2. CNF; in particular 36 isolates were CNF2-positive, eight were CNF2-positive and two isolates were positive to both CNF1 and CNF2.

### Table 2

| E. coli (%) | Amp | Ot | Stx | Sxt | C | 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|

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

*Percentage of resistant (R), intermediate susceptible (I) and susceptible (S) E. coli isolates.

*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), cytotoxic-thall distending toxins (CDT-I, CDT-II, CDT-III and CDT-IV), and F17 fimbriae family (F17a, F17b, F17c and F17d).

*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), cytotoxin-producing E. coli (AEEC) 4.1; EHEC 2.7 Stx1; eae; Ehly 4

## Table 3

| E. coli type | Frequency (%) | Virulence factors* | No. of 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. Indeed, water buffalo NTEC frequency appeared comparable to those observed for the newer molecules.

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 alarming 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 medicinal 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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