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Isolation, molecular characterization and antibiotic resistance of Enterotoxigenic *E. coli* (ETEC) and Necrotoxigenic *E. coli* (NTEC) from healthy water buffalo

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ABSTRACT

The present study was undertaken to detect the prevalence, virulence gene profile and antibiotic resistance of Enterotoxigenic *Escherichia coli* (ETEC) and Necrotoxigenic *E. coli* (NTEC) in healthy water buffalo in West Bengal. Out of the 363 *E. coli* isolates from 165 faecal samples, 18 (4.95%) and 7 (1.9%) isolates were found to possess genes for ETEC (*LT* or *STa* or *STb*) and NTEC (*cnf1* or *cnf2*), respectively in PCR. Among the 18 ETEC isolates, 13 (72.22%), 12 (66.67%), and 1 (5.56%) isolates were found to harbour *STa*, *LT*, and *STb* genes respectively. However, among the 7 NTEC isolates, 3 (42.85%) and 4 (57.14%) were detected to possess *cnf1* and *cnf2* genes, respectively. Further, among the five isolates possessing the EAST 1 gene, four were detected with the *STa* gene and one (1) with the *LT* gene. However, the majority of the F41 strains possessed *ST* genes, and F5 strains harboured both LT and ST. In addition, one out of three *cnf1* NTEC isolates was detected to harbour the *papC* gene and all the four *cnf2* NTEC isolates contained the *cdt* gene. The ETEC isolates belonged to 11 different serogroups (O11, O53, O71, O84, O88, O103, O112, O120, O128, O153, and O158), while, four NTEC isolates belonged to the OUT serogroup and the remaining 3 were disseminated among the O2, O4, and O6 serogroups. The RAPD of the ETEC isolates produced different electrophoretic profiles. The antibiotic resistance of ETEC and NTEC isolates was observed most frequently to amikacin (56%), kanamycin (44%),

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gentamicin (40%), neomycin (36%). The study thus revealed water buffalo as a reservoir of multi drug resistant ETEC/NTEC for the first time in India.

Key words: antibiotic resistance, water buffalo, *E. coli*, India, enterotoxigenic, necrotoxigenic, polymerase chain reaction

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are described as *E. coli* strains that elaborate at least one of the two defined groups of enterotoxins, heat-stable (ST) and heat-labile toxin (LT) and they cause traveller's diarrhoea and children's diarrhoea, specially in developing countries (LIAQAT, 2012). Among the two major subtypes of ST, known as STa and STb, cattle are reported to be a reservoir of STa (BLANCO et al., 1993). However, among the two major LT subtypes, LT-I is expressed by *E. coli* strains that are pathogenic to both human and animals, and LT-II is primarily expressed by animal *E. coli* isolates and has not been found to be associated with clinical disease (QADRI et al., 2005). In addition, ETEC strains may possess several adhesins such as F5 and F41, and another toxin known as Enteroaggregative *E. coli* heat stable enterotoxin (EAST1) (CHOI et al., 2001). The ETEC strains are considered to be an important world-wide cause of enteric disease in domestic animals, particularly in cow calves, buffalo calves, piglets and lambs, as well as diarrhoea in humans, particularly in children and travellers (USEIN et al., 2009; BANDYOPADHYAY et al., 2011; BORRIELLO et al., 2012).

Necrotoxigenic *E. coli* (NTEC) are able to produce two kinds of cytotoxic necrotising factors (CNF1 and CNF2), causing urinary tract infection (UTI), septicaemia and diarrhoea in humans and animals (ORDEN et al., 2002). The NTEC isolates exuding CNF1 are commonly observed to cause human extra-intestinal infections, whereas CNF2 containing NTEC have been isolated from healthy (ORDEN et al., 2002) as well as diarrhoeic calves (BLANCO et al., 1998) and buffaloes (BORRIELLO et al., 2012). Further, the *cnf1* gene is associated with a pathogenicity island containing a *pap* gene cluster, specially *papC* encoding for a transporter protein that helps in pathogenesis. *cnf 2* is associated with the *cdt* gene encoding the cytolethal distending toxin, causing eukaryotic cell cycle block at the G2 stage, prior to mitosis (VAN BOST et al., 2003; SAMANTA, 2013).

In India, a few reports are available on detection of ETEC from diarrhoeic calves (KUMAR et al., 1982; PANDA and PANDA, 1987), mithun (RAJKHOWA et al., 2009), lambs (BANDYOPADHYAY et al., 2011) and as well as from humans (KANG et al., 2001). However, to the authors' best knowledge no studies to detect ETEC/NTEC in healthy or diarrhoeic buffaloes have been conducted so far. So, the present study was designed to discover the prevalence of ETEC, NTEC with their virulence gene profile and antibiogram in healthy water buffaloes in West Bengal, a major buffalo rearing state in India.

Materials and methods

A total of 165 faecal samples from water buffaloes were collected randomly from seven districts (Bankura, North 24 parganas, Murshidabad, Maldah, Hooghly, Nadia and Burdwan) of West Bengal, India. The samples were collected from apparently healthy water buffalo of all ages, sexes and breeds.

The faecal samples were collected into sterile vials directly from the rectum using a sterile cotton swab stick (HiMedia, India). All the samples collected were brought to the laboratory in ice packs and processed by standard isolation and identification techniques as described earlier (MAHANTI et al., 2013).

For PCR based detection of virulence genes from all the *E. coli* isolates, DNA was extracted as per the previously described method of WANI et al. (2003).

The detection of virulence factors characteristic of ETEC like *STa*, *STb, LT* and NTEC characterized by *cnf1*, *cnf2* was performed by PCR using the oligonucleotide primers (Genetix Biotechnology Asia Private Limited) and cycle conditions described earlier (BORRIELLO et al., 2012; BLANCO et al., 1998). The amplified DNA products of all the PCR reactions were analysed by gel electrophoresis in 1-2% agarose containing ethidium bromide (0.5 µg mL^{-1}) as per the standard protocol (SAMBROOK and RUSSEL, 2001).

All the ETEC isolates were subjected to PCR for detection of F5, F41 and EAST-1 genes as described earlier (CHOI et al., 2001) and all the NTEC isolates were screened for the adhesins like *papC* and *cdt* by PCR as previously reported (VAN BOST et al., 2003).

After confirmation by PCR all the ETEC/NTEC isolates were sent for O- serogrouping to National *Salmonella* & *Escherichia* Centre, Central Research Institute, Kasuli, HP, India.

The molecular characterization of all the ETEC isolates was done by RAPD-PCR using a single primer 1254 (CCGCAGCCAA, Genetix Biotechnology Asia Private Limited) as per the protocol of PACHECO et al. (1997). The PCR products were then electrophoresed in 1% (W/V) agarose gel containing ethidium bromide (0.5 µg/mL) (SAMBROOK and RUSSEL, 2001).

All the confirmed ETEC and NTEC isolates were tested for their sensitivity and resistance to different antibiotics by the disc diffusion method (CLSI, 2008). The antibiotics used were amikacin (30 µg), gentamicin (30 µg), kanamycin (30 µg), neomycin (30 µg), oxytetracycline (30 µg), co-trimoxazole (25 µg), ceftazidime (30 µg), levofloxacin (5 µg), cefepime (30 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), enrofloxacin (5 µg), pefloxacin (5 µg), amoxycillin (25 µg), chloramphenicol (30 µg), cefuroxime (30 µg) and norfloxacin (10 µg) (Hi Media, India).

Results

From the 165 faecal samples of apparently healthy water buffaloes examined, a total of 363 isolates were identified as *E. coli* on the basis of staining property, colony characteristics, and standard biochemical reaction.

Of the 363 strains of *E. coli* isolated from buffalo, 18 (4.95%) and 7 (1.9%) isolates were found to possess some genes for ETEC (*LT* or *STa* or *STb*) and NTEC (*cnf1* or *cnf2*), respectively. Among the 18 ETEC isolates, 13 (72.22%), 12 (66.67%), and 1 (5.56%) isolates were found to harbour *STa*, *LT*, and *STb* genes respectively (Table 1). However, among the 7 NTEC isolates, 3 (42.85%) and 4 (57.14%) were detected to possess *cnf1* and *cnf2* genes, respectively (Table 2).

Table 1. Occurrence of different genotypes, serogroups and RAPD electrophoretic profile in the ETEC isolated from healthy buffaloes in West Bengal, India

Genotype	Serogroup	RAPD Electrophoretic profile
STa	O120	6
	O103	4
	O103	2
	O153	10
STa, LT	O88	11
	O88	9
STb, LT	O84	12
	O71	13
STa, F41	O112	15
STa, LT, F41, K99	OUT	13
	O53	12
LT, K99	O11	13
LT, K99, EAST1	O158 (2)	14
STa, EAST1, K99	O120	13
	OUT	13
STa, LT, EAST1	O128	14
	O128	12

Table 2. Occurrence of different genotypes and serogroups of the NTEC isolated from healthy buffaloes in West Bengal, India

Genotype	Serogroup (s)
CNF1, *papc*	OUT
CNF1	OUT (2)
CNF 2, *cdt*	O2, O4, O6, OUT

We also detected the EAST-1, F5 and F41 genes by PCR in 5 (27.77 %), 4 (22.22 %), and 3 (16.66 %) ETEC isolates, respectively along with enterotoxin genes (Table 1). Among the five isolates possessing the EAST 1 gene, four were with the *STa* gene and one was with the *LT* gene, respectively. Further, the majority of the F41 strains also possessed *ST* genes, whereas, F5 strains harboured both LT and ST.

One out of three *cnf1* NTEC isolates was also found to harbour the *papC* gene and all the four *cnf2* possessing NTEC isolates contained the *cdt* gene (Table 2).

The ETEC isolates belonged to 11 different serogroups (O11, O53, O71, O84, O88, O103, O112, O120, O128, O153, O158) and two of them were untypable (UT). Four NTEC isolates belonged to the OUT serogroup and the others (3) were O2, O4, and O6 serogroups.

All the ETEC strains were typeable with primer 1254 and they produced amplified fragments ranging from 200bp to 2000 bp. The RAPD produced different electrophoretic profiles for each of the isolates, comprised of 2-15 bands (Table 1). However, no profile similarity was observed between the strains isolated from the same area even between the same serogroups.

The antibiotic resistance of ETEC and NTEC isolates was observed most frequently to amikacin (56%), kanamycin (44%), gentamicin (40%), neomycin (36%), oxytetracycline (28%), and co-trimoxazole (24%). No resistance was observed in the case of ceftazidime, levofloxacin and cefepime. The antimicrobial agents against which higher sensitivity was found were ciprofloxacin (84%), ceftriaxone (84%), enrofloxacin (80%), pefloxacin (76%), amoxicillin (72%), chloramphenicol (68%), cefuroxime (68%), and norfloxacin (60%). Multi-drug resistance was observed in 11 (44%) strains.

Discussion

Ruminants act as reservoirs of major diarrhoeagenic *E. coli* pathotypes, such as shiga toxin producing *E. coli* (STEC), which may enter the human food chain (WANI et al., 2004). However, a ruminant such as the buffalo has not been established as a reservoir of ETEC/NTEC, another major diarrhoeagenic *E. coli* pathotype. So, the present study was undertaken to detect the prevalence, virulence gene profile and antibiotic resistance pattern of ETEC/NTEC in healthy water buffaloes.

The study detected 18 (4.95%) ETEC isolates from buffalo as positive for some of the enterotoxin genes in PCR. Previously, a higher prevalence of ETEC (10%) was found in water buffalo by ECHEVERRIA et al. (1978) in the Philippines. Meanwhile, a much lower prevalence rate of ETEC (1.8%) was observed in diarrhoeic buffalo in Italy (BORRIELLO et al., 2012). In India, the occurrence of ETEC associated with calf diarrhoea was reported earlier (KUMAR et al., 1982; PANDA and PANDA, 1987). A similar prevalence rate of ETEC (5.5%) was observed in other mammalian species, such as diarrhoeic mithun calves in Nagaland (RAJKHOWA et al., 2009). However, a higher prevalence rate (9%) was observed

in diarrhoeic lambs in Arunachal Pradesh (BANDYOPADHYAY et al., 2011). There is no report of ETEC prevalence in Indian buffaloes, with or without diarrhoea, to compare the present result.

Among the 18 ETEC isolates, 13 (72.22%), 12 (66.67%), and 1 (5.56%) isolate was found to harbour *STa*, *LT*, and *STb* genes, respectively. In contrast, all the ETEC isolates from diarrhoeic buffalo in Italy possessed only *LT* without any *ST* gene (BORRIELLO et al., 2012). However, in diarrhoeic buffalo calves, STa enterotoxin was detected in 14.7% of *E. coli* isolates without any LT enterotoxin in Brazil, using the infant mice inoculation technique (RIBEIRO et al., 2008). In India, similarly, RAJKHOWA et al. (2009) found more *ST* gene possessing ETEC isolates than *LT* in diarrhoeic mithun calves. However, BANDYOPADHYAY et al. (2011) found equal numbers of *LT* and *ST* genes (17out of 22) in ETEC isolates from diarrhoeic sheep.

Out of the 363 *E. coli* isolates, 7 (1.9%) were found to possess some of either gene for NTEC (*cnf1* or *cnf2*). However, a higher prevalence rate of NTEC (20.9%) was observed in diarrhoeic buffaloes in Italy (BORRIELLO et al., 2012). In healthy cattle higher prevalence rates of NTEC have also been reported, such as 9.9% and 6.1% in Spain and Poland, respectively (ORDEN et al., 2002; OSEK, 2001). In India, no studies related to NTEC prevalence in animals are available to compare the present data. However, NTEC was reported to be associated with acute diarrhoea in children (12%) in South India (DE RYCKE et al., 1999).

Among the 7 NTEC isolates, *cnf2* (4, 57.14%) was more frequently observed than *cnf1* (3, 42.85%). Similarly, NTEC isolates with more *cnf2* (83%) than *cnf1* (22%) were isolated in diarrhoeic *buffaloes* in Italy (BORRIELLO et al., 2012). Further, *cnf 1* alone has been found to be responsible for human diarrhoea worldwide (BEKAL et al., 2003). However, in India both *cnf 1* and *cnf 2* are found to be associated with childhood diarrhoea (KAVITHA et al., 2010).

The present study detected the EAST1 gene in the highest frequency (27.77%) in the ETEC isolates from buffalo, the majority of which were associated with *STa* (4 out of 5). Similarly, CHOI et al. (2001) also detected the maximum number of ETEC isolates in piglets possessing the EAST1 gene (63%), along with *STa*. Our present finding of the F5 gene in ETEC isolates is also supported by earlier work in animal ETEC strains (BEKAL et al., 2003). Further, the majority of the F41 strains possessed *ST,* and F5 strains harboured both *LT* and *ST*. In contrast, CHOI et al. (2001) found F41 strains without any enterotoxin gene and F5 strains with only *ST* in piglets in Korea.

Further characterization of NTEC strains from buffaloes revealed the presence of *papC* and *cdt* genes in the isolates possessing *cnf1* and *cnf2* genes, respectively (Table 2). Similarly, VAN BOST et al. (2003) also found *cnf1* possessing NTEC isolates with or without the *papC* gene, and *cnf2* containing NTEC isolates with the *cdt* gene from cattle, dogs and humans. However, MAINIL et al. (1999) reported more PAP producing NTEC 1

isolates (63-81%) than the negligible amount of NTEC 2 isolates (3%) from animals and human. In another study, they also detected *cdtB* gene more frequently in *cnf1* isolates (83%) than *cnf2* (4-15%) from humans and animals (MAINIL et al., 2003).

The ETEC isolates in the present study belonged to the O11, O53, O71, O84, O88, O103, O112, O120, O128, O153, O158, and OUT serogroups. Similarly, TOMA et al. (2003) and ALAM et al. (2006) also detected serogroups O128 and O153 as ETEC from diarrhoeic humans in Japan, and aquatic sources in Bangladesh, respectively. In India, BANDYOPADHYAY et al. (2011) also found ETEC serogroups O120 and OUT in diarrhoeic sheep in Arunachal Pradesh.

Four NTEC isolates belonged to the OUT serogroup and the others were O2, O4, and O6. The serogroups (O2, O4, O6) apart from OUT were earlier reported as NTEC from humans and animals (MAINIL et al., 1999; BLANCO et al., 1992).

In RAPD analysis of ETEC isolates, a similar type of heterogeneity was observed in the ETEC strains isolated from children in Brazil (MANGIA et al., 2004). A finding of different electrophoretic profiles, even within the same serogroup, was also detected earlier in non-O157 *E. coli* isolates (KRUGER et al., 2006).

The antibiotic resistance of ETEC and NTEC isolates was observed most frequently to amikacin (56%), kanamycin (44%), gentamicin (40%), neomycin (36%), oxytetracycline (28%), and co-trimoxazole (24%). Similarly, NIZZA et al. (2010) found absolute resistance of haemolytic *E. coli* isolated from buffalo calves to neomycin and oxytetracycline. Susceptibility to norfloxacin and chloramphenicol was also found earlier in *E. coli* isolates from buffalo calves in Brazil (RIBEIRO et al., 2000).

Thus the present study revealed for the first time the moderate occurrence of multi drug resistant ETEC or NTEC in healthy buffaloes in the eastern part of India, which may pose a serious threat to human health.

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SAŽETAK

Istraživanje je provedeno s ciljem da se ustanovi prevalencija, geni za virulenciju i otpornost na antibiotike enterotoksigenih (ETEC) i nekrotoksigenih (NTEC) sojeva bakterije *Escherichia coli* izdvojene iz zdravih vodenih bivola iz Zapadnog Bengala. Od 363 izolata *E. coli* iz 165 uzoraka fecesa, 18 (4,95%) je posjedovalo neki od gena za ETEC (*LT* ili *STa* ili *STb*), a sedam (1,9%) izolata imalo je gene za NTEC (*cnf1* ili *cnf2*) dokazane PCR-om. Od 18 izolata ETEC, 13 (72,22%) je imalo gen *STa*, 12 (66,67%) gen *LT* i jedan (5,56%) gen *STb*. Međutim, od sedam NTEC izolata, tri (42,85%) su imala gen *cnfl,* a četiri (57,14%) gen *cnf2.* Nadalje, od pet izolata koji su posjedovali gen *EAST* 1, četiri su imala gen *Sta*, a jedan gen *LT*. Većina sojeva F41 posjedovala je gen *ST*, a sojevi F5 i *LT* i *ST* gen. Nadalje, jedan od triju *cnf1* NTEC izolata imao je i gen *papC*, a sva četiri izolata *cnf2* NTEC sadržavala su gen *cdt*. Izolati ETEC pripadali su u 11 različitih seroloških skupina (O11, O53, O71, O84, O88, O103, O112, O120, O128, O153 i O158). Četiri izolata NTEC pripadala su serološkoj skupini OUT, a preostala tri skupinama O2, O4 i O6. RAPD izolata ETEC davao je različite elektroforetske profile. Izolati ETEC i NTEC najčešće su bili otporni na amikacin (56%), kanamicin (44%), gentamicin (40%) i neomicin (36%). Ovim istraživanjem prviput je u Indiji ustanovljeno da su vodeni bivoli rezervoar multiplo rezistentnih sojeva ETEC/NTEC.

Ključne riječi: otpornost na antibiotike, vodeni bivol, *E. coli*, Indija, enterotoksigeni sojevi, nekrotoksigeni sojevi, lančana reakcija polimerazom