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Detection of toxin genes and antibiogram pattern in *Escherichia coli* isolates from sheep meat on Indian market

Mahendra Mohan Yadav*, Ashish Roy, Rakesh Sharda, and Gitanjali Arya

Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand, Gujarat State, India

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ABSTRACT

Isolates (49) of *Escherichia coli* were isolated from 100 samples of mutton collected from dressed sheep carcasses by excision method. The isolates were typed into 12 different 'O' serogroups. The most frequent serovar isolated was O101, followed by O152, O76, O23, O139, O88, O110, O126, O8, O9, O15 and O133; two isolates were untypable while one was rough. All the isolates of *Escherichia coli* were screened for the presence of VT1, VT2, LT and ST genes by PCR and *in vitro* antibiotic resistance pattern. Nine (60.0%) and six (40.0%) isolates were found positive for VT1 and VT2 genes, respectively. Five (33.33%) were found positive for both the VT1 and VT2 genes. Four (26.67%) isolates were positive for LT gene, whereas only one (6.67%) isolate was found positive for ST gene. The percentage of isolates resistant to antibacterial agents was recorded as follows: sulphadiazine (93.33%), cephaloridine (80.00%), cephalexin (33.33%), penicillin G, ceftiofur and norfloxacin, carbenicillin and enrofloxacin (26.67% each), and oxytetracycline and amoxycillin (20.00% each).

Key words: antibiogram, PCR, *Escherichia coli*, toxins, sheep meat

Introduction

Microbes in meat, especially those causing food borne diseases in human beings, have recently become a matter of great public health concern. India could earn a considerable amount of foreign exchange through meat exports, but the major constraints are its poor quality and high level of microbial load.

The importance of enterotoxigenic *Escherichia coli* (ETEC) as a causative agent in childhood diarrhea is well recognized (TARDELLI et al., 1991). Two groups of plasmid-encoded enterotoxins have been described, one is heat-labile (LT) toxin and the other is

*Contact address:

Mahendra Mohan Yadav, Ph.D., Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand 388 001, Gujarat State, India, Phone: +91 990 4238 241; Fax: +91 2692 261 201; E-mail: mmy_ 20@rediffmail.com

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heat-stable (ST) toxin. Shiga-like toxin-producing *Escherichia coli* (STEC), also known as Verocytotoxin producing *Escherichia coli* (VTEC), is recognized as another important food borne pathogen responsible for sporadic cases as well as for serious outbreaks worldwide.

Domestic animals, especially sheep and cattle, are the main reservoirs and sources of *E. coli* infection for human beings (SIDJABAT-TAMBUNAN et al., 1998). Contaminated meat and meat products, dairy products, vegetables, drinking water and swimming pools have been recognized as main vehicles for spreading the infection to humans. To reduce the impact of toxigenic isolates, their epidemiology must be fully established. Epidemiological studies would be greatly facilitated by the availability of a technique, such as PCR, which reliably detects low numbers of pathogens in food, water, and environmental materials.

The increasing demand for animal proteins, like meat and meat products, has increased the load of slaughterhouses resulting in inadequate attention being paid to the hygienic aspect of meat production. Meat being a nutrient-rich substrate can support the growth of a wide range of micro-organisms. Ever since the identification of ETEC and VTEC as a human pathogen, *E. coli* have received much attention as a potential public health threat due to the morbidity and mortality rates associated with outbreaks and sporadic cases of human illness. Despite the fact that India has the largest population of livestock, no systematic study of prevalence of ETEC and VTEC in end products obtained from these animals has been carried out. Bearing in mind the impact this bears on both the economy and the public health, this research has been undertaken with the aim to investigate the prevalence of STEC and ETEC in dressed sheep meat, and their antibiotic resistant pattern.

The indiscriminate use of antibiotics has led to the emergence of antimicrobial resistance in various isolates of bacteria (GHOSH et al., 2003). It is well documented that drug resistance could be transferred between related bacteria, such as *E. coli* and *Salmonella*, via R-factor, both *in vitro* and *in vivo* (VERMA, 1988). Evolution of such resistant bacteria may create problem in treatment of acute infections in man and animals. The antimicrobial agents are of great value for devising curative measures against bacterial infections. However, the frequency of R-factor and its transmissibility in *E. coli* posses a serious problem in treating such infections.

Thus, with great expansion of sheep meat industry, *E. coli* has emerged as a problem of economic concern to all phases of meat industry - from production to marketing to consumer health significance, to the clinicians - due to emergence of multiple drug resistant bacterial isolates, and to veterinarians due to reservoirs of infection. Sheep meat or its products are one of the common dietary items of the people of Central and Western

India. Consequently, the present study was undertaken to detect LT, ST, VT1 and VT2 genes, and *in vitro* antibiogram of the *E. coli* isolates from sheep meat.

Materials and methods

Samples from one hundred dressed sheep carcasses were aseptically collected from the local meat shops located in Mhow town. Samples were taken randomly from five retail market shops on every Sunday. Not more than five samples were taken at a time, one each from five different meats shops. Isolation of *E. coli* was carried out within an hour after collection of samples. The meat samples were collected as per the procedure recommended by International Commission on Microbiological Specification for Food (ANONYMOUS, 1978) by excision method (destructive method), taking approximately 10g samples from each of the five sites viz., neck, right fore limbs and hind limbs, and left fore limbs and hind limbs (AMIN and BORAH, 2002). These samples were transferred to the laboratory in sterile stomacher bags on ice**.** Samples that were above 10 °C upon arrival at the testing laboratory were discarded.

Table 1. The primer pairs for LT, ST, VT1 and VT2 genes

Primers	Sequences (5'- 3')	Target Gene	Size of amplified product (bp)	Reference
LT1 (F) LT2 (R)	AGC AGG TTT CCC ACC GGA TCA CCA GTG CTC AGA TTC TGG GTC TC	LT	132	NISHIKAWA et al. (2002)
MicroST1 (F) MicroST2 (R)	TTT ATT TCT GTA TTG TCT TT ATT ACA ACA CAG TTC ACA G	ST	171	NISHIKAWA et al. (2002)
VT1a (F) VT1b(R)	GAA GAG TCC GTG GGA TTA CG AGC GAT GCA GCT ATT AAT AA	VT1	130	POLLARD et al. (1990)
VT1 (F) VT2(R)	TTT ACG ATA GAC TTC TCG AC CAC ATA TAA ATT ATT TCG CTC	VT2	228	NISHIKAWA et al. (2002)

F - forward primer, R - reverse primer

On arrival in the laboratory, the samples were immediately thawed to room temperature and those collected from different sites of same carcass were pooled in one stomacher bag. The pooled sample was then homogenized at 8000 rpm for 2 min. in 450 mL of normal saline solution (NSS) to obtain 1:10 dilution, which was subsequently used as an inoculum for isolation of *E. coli*.

Table 2. Steps and conditions of thermo cycling for PCR

Steps	Temp./Time (LT1 and LT2 primer)	Temp./Time (micro ST1 and micro ST2 primer)	Temp./Time (VT1 and VT2 primer)	Temp./Time (VT1a and VT1b primer)
Initial denaturation	-	-	-	94 °C 5 min
Denaturation	94 °C 30 sec	94 °C 30 sec	94 °C 30 sec	94 °C 2 min
Annealing	45 °C 1 min	45 °C 1 min	45 °C 1 min	55 °C 1 min
Extension	72 °C 1.5 min	72 °C 1.5 min	72 °C 1.5 min	72 °C 1 min
Final extension	-	-	-	72 °C 7 min
Cycles	25 cycles	25 cycles	25 cycles	35 cycles

For isolation of *E. coli* MacConkey broth (single strength), MacConkey agar (MCA) and Eosin-methylene blue (EMB) agar were used as enrichment, differential and selective medium, respectively (ANONYMOUS, 1978). The enrichment was done at 37 °C for 12-18 hr, while the MCA and EMB agar were incubated at 37 °C for 24 hr. The smooth and moist colonies with metallic sheen on EMB agar were randomly sub cultured and purified by limiting dilution. The isolates were identified on the basis of their cultural, morphological and biochemical characteristics (BARROW and FELTHAM, 1993). They were serotyped at National Salmonella and Escherichia Typing Centre, Central Research Institute, Kasauli, India. Twelve randomly selected *E. coli* isolates, each representing a serovar, one rough and 2 untypable isolates were further used in the present study.

Detection of toxigenic genes by PCR. Total bacterial DNA was prepared as described by WILSON (1987). PCR was carried out in a final volume of 15 µL containing 7.5 µL of 2 × PCR Master mix (4 mM $MgCl_2$, 0.4 mM of each dNTPs, 0.05 units/mL of Taq DNA polymerase, 150 mM tris HCl PCR buffer) (Fermentas, Life Sciences), 0.50 µL of each

primer (10 pmole/μL), DNAse free distilled water and 1.0 μL of template DNA. The primer pairs and the thermal cycling program for PCR used in this study are shown in Table 1 and Table 2, respectively. The analysis of the PCR products was performed by 2.0% horizontal agarose gel electrophoresis stained by ethidium bromide and observed under UV light.

Antimicrobial susceptibility test. The *in vitro* sensitivity of *E. coli* isolates towards 11 different antibiotics was determined by disc diffusion method of BAUER et al. (1966) on Mueller-Hinton agar (Bio Mark Ltd, Pune, India). A loopful of the growth from slant was inoculated in Brain Heart Infusion (BHI) broth and incubated at 37 °C for 3 to 5 h. The opacity of broth tube was matched with McFarland's tube N^{o} 0.5 (1.5×10^6 organisms/mL). A sterile cotton swab was dipped into the broth culture; excess of the bacterial suspension was removed by pressing and rotating the swab against the inner walls of the test tube. Streak were applied across the entire agar surface of the plate with the swab three times, the plate being turned for 60° between each streaking. The surface of preincubated and sterile Muller-Hinton agar (Bio Mark Ltd, Pune, India) petri plate was kept at room temperature for 30 min. to allow the inoculum to be adsorbed on the surface. Antibiotic sensitivity discs (Hi Media Ltd, Mumbai, India) were placed with the help of flamed forceps on the plates at equal distance and sufficiently separated from each other. The plates were incubated overnight at 37 °C. Antibiotics used in the present study were amoxycillin (25 μg), carbenicillin (100 μg), ceftiofur (0.2 μg), cephaloridine (30 μg), cephalexin (30 μg), enrofloxacin (10 μg), norfloxacin (10 μg), oxytetracycline (30 μg), penicillin G (10 units), and sulphadiazine (300 μg).The antibiotic discs were procured from M/s Hi Media Labs (Mumbai, India). The results were interpreted according to the instructions of the manufacturer.

Results

Detection of LT and ST genes. Only one *E. coli* isolate (4.16%) belonging to serogroup O88 harbored ST gene (171bp), and 4 isolates belonging to O8, O101, O133 and O139 serogroups, were found positive for LT gene (132 bp) by PCR test (Table 3).

Detection of VT1 and VT2 genes. Of the 15 isolates of *E. coli*, 9 (60.0%) were positive for VT1 gene. These isolates belonged to nine different 'O' serogroups, viz., O8, O76, O88, O101, O110, O126, O133, O139 and O152. Similarly 6 isolates (40.0%) belonging to serogroup O9, O101, O110, O126, O133 and O139 were found positive for VT2 gene (Table 3). Five (33.33%) *E. coli* isolates of the serogroup O101, O110, O126, O133 and O139 carried both VT1 and VT2 genes.

Table 3. Results of PCR amplification of toxigenic genes in *E. coli* isolates

Isolate Nº	Serogroup	Heat labile	Heat stable	Verotoxin 1	Verotoxin 2
1.	O8	+	-	+	-
2.	O9	-	-	-	+
3.	O15	-	-	-	-
4.	O23	-	-	-	-
5.	O76	-	-	+	-
6.	O88	-	+	+	-
7.	O101	+	-	+	+
8.	O110	-	-	+	+
9.	O126	-	-	+	+
10.	O133	+	-	+	+
11.	O139	+	-	+	+
12.	O152	-	-	+	-
13.	UT	-	-	-	-
14.	UT	-	-	-	-
15.	R	-	-	-	-

UT - untypable, R - rough

Table 4. Number and percent of *E. coli* isolates resistant to antimicrobial drugs

Antibiotic	Resistant isolates of *E. coli*	
	Nº	%
Am	3	20.00
Cb	4	26.67
Fur	4	26.67
Cp	5	33.33
Cr	12	80.00
Ex	4	26.67
Nx	4	26.67
O	3	20.00
P	4	26.67
S	14	93.33

Am - amoxycillin; Cb - carbenicillin; Fur - feftiofur; Cp - cephalexin; Cr - cephaloridine; Ex - enrofloxacin; Nx - norfloxacin; O - oxytetracycline; P - penicillin G; S - streptomycin.

Antimicrobial drug resistance to E. coli isolates. The number and percent of *E. coli* isolates resistant to various antimicrobial drugs are shown in Table 4. Among the 15 isolates of *E. coli* tested for resistance against various antibiotics all the isolates (100.0%) were found to be resistant to erythromycin and streptomycin, followed by sulphadiazine (95.84%) and cephaloridine (87.50%). Moderately high resistance was detected towards cephalexin (41.69%), penicillin G (37.60%), ceftiofur and norfloxacin (33.36% each), enrofloxacin (27.40%) and carbenicillin (25.30%). The isolates were least (16.70%) resistant to the antibiotics amoxycillin and oxytetracycline. Multiple drug resistance was demonstrated in ten isolates, showing simultaneous resistance to 2 to 10 antibacterial agents. Out of 15 *E. coli* isolates, one isolate (6.67%) was sensitive to all the antibacterial agents. Three (20.0%) isolates were resistant to one antibacterial agent. Four (26.67%) isolates were resistant to two antibacterial agents, two (13.33%) isolates were resistant to three antibacterial agents, and one isolate (6.67%) was resistant to seven antibacterial agents, while three (20.0%) isolates were resistant to all the 10 antibacterial agents. Five different multiple drug resistance patterns were recorded.

Discussion

In the present study 4 (26.67%) isolates of *E. coli* were found positive for LT gene. Only one (6.67%) isolate of O88 serogroup was found positive for ST gene. KAPOOR et al. (2002) also detected ST enterotoxin in *E. coli* from meat, while ORSKOV et al. (1977) reported that isolates belonging to O88 produced ST enterotoxin. Thus our results are in concurrence with the above reports.

In the present investigation VT1 and VT2 genes were detected in 60.0% and 40.0%, respectively, of sheep meat samples. SIDJABAT-TAMBUNAN and BENSINK (1997) found that sheep have about 40.6% carriage rate of VTEC in their faeces. They further reported that 28.0% sheep carcase samples harboured VT1 gene and 6.0% VT2 gene in winter season, while in summer season the carriage rate was 74.0% and 9.0%, respectively for these two genes (SIDJABAT-TAMBUNAN et al., 1998). HAZARIKA et al. (2004) reported 25.0% of *E. coli* isolates to be Verotoxin producing. Thus, our finding is in partial agreement with the finding of above authors. However, comparison is difficult because of difference in modes of evaluation, hygienic standards followed in abattoirs, and geoclimatic conditions. Higher frequency of isolation of VTEC in the present study from dressed sheep meat might be due to contamination from the intestinal contents of a slaughtered animal since the intestinal tract of sheep and cattle has been shown to be a major reservoir of VTEC (KUDVA et al., 1997). During a carcase dressing faecal contamination of the carcasses can occur either by direct contact with animal's fleece, legs and/or hoofs or via the worker.

In the present study, sensitivity of *E. coli* isolates was studied *in vitro* against 10 antibacterial agents. The isolates were highly resistant to sulphadiazine (93.33%), followed by cephaloridine (80.00%), cephalexin (33.33%), penicillin G, ceftiofur and norfloxacin, carbenicillin and enrofloxacin (26.67% each), and oxytetracycline and amoxycillin (20.00% each). BORO et al. (1983) observed 92.30% *E. coli* isolates resistant to sulphadiazine, while TAKALE et al. (1994) reported 8.33% and 91.66% isolates sensitive to streptomycin and tetracycline, respectively. These findings are in corroboration with our findings. BANERJEE et al. (2001) recorded that 41.67% *E. coli* isolates were resistant to cephalexin, which is in partial agreement with our result. AHMAD et al. (2004) reported that 18.18% isolates were resistant to amoxycillin which is in close agreement with our findings. MOTINA and YADAVA (2005) observed 41.37% *E. coli* isolates resistant to norfloxacin and penicillin, which was in partial agreement with our results.

Multiple drug resistant isolates of *E. coli* isolates from animals have been reported worldwide. Use of antimicrobials in livestock production is suspected to significantly contribute to multiple drug resistance in species of bacteria, which are common to humans and animals (ACAR and ROSTEL, 2001). Multiple drug resistant species of enterobacteriaceae are hence creating a constant need for new antibiotics worldwide.

Conclusions

The present investigation was aimed to detect toxigenic genes (VT1, VT2, LT and ST) by PCR, and antimicrobial drug sensitivity in *E. coli* isolates from sheep meat. The study concludes that *E. coli* isolates from sheep meat harbored VT1, VT2, LT and ST gene. From the standpoint of meat hygiene, the level of LT, VT1 and VT2 positive *E. coli* contamination of sheep carcasses in slaughterhouses are relatively high. High levels of these toxin genes suggest that either diseased or carrier animals were used for slaughtering or that hygienic conditions in the slaughterhouses and retail shops are of low standards. The maintenance of slaughter hygiene is consequently of central importance in meat production. In view of high prevalence of LT, VT1 and VT2 sheep should not be slaughtered at all or should be slaughtered on a separate slaughtering line. With highly soiled animals, it is possible that high level of LT, VT1 and VT2 were detected. Presence of multidrug resistant isolates causes alarm and underlines the necessity as to a careful choice and a cautious use of antibiotics in *E. coli*. Furthermore, molecular characterization of fimbrial genes is required in order to explore the significance of such genetic combinations.

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SAŽETAK

Ukupno je 49 izolata bakterije *Escherichia coli* bilo izdvojeno iz 100 uzoraka mesa uzetih ekscizijom iz obrađenih ovčjih trupova. Izolati su bili tipizirani i svrstani u 12 različitih seroloških skupina O. Najčešće je bio izdvojen serovar O101, a zatim serovarovi O152, O76, O23, O139, O88, O110, O126, O8, O9, O15 i O133. Dva izolata ostala su netipizirana, a jedan je bio s hrapavim kolonijama. Svi su izolati bili pretraženi lančanom reakcijom polimerazom na prisutnost gena VT1, VT2, LT i ST te im je *in vitro* određena osjetljivost prema antimikrobnim tvarima. Devet (60,0%) izolata bilo je pozitivno za gen VT1, a šest (40,0%) za VT2. Pet (33,33%) ih je bilo pozitivnih za oba gena, VT1 i VT2. Četiri izolata (26,67%) bila su pozitivna za gen LT, dok je samo jedan (6,67%) bio pozitivan za gen ST. Na sulfadiazin je bilo otporno 93,33% izolata, na cefaloridin 80,00%, na cefaleksin 33,33%, na penicilin G, ceftiofur, norfloksacin, karbenicilin i enrofloksacin 26,67% te na oksitetraciklin i amoksicilin 20,00% izolata.

Ključne riječi: antibiogram, PCR, *Escherichia coli*, toksini, ovčje meso