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Detection of Heat-Stabile and Heat-Labile Enterotoxins of *Escherichia coli* Strains Isolated from Healthy Animals

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Abstract: In this study, *Escherichia coli* strains isolated from feces of 12 various healthy animals (sheep, cow, dog, quail, cattle, ostrich, lamb, gull and chicken, Turkey, cat and pigeon) were examined for heat Stabile enterotoxin (ST) and heat Labile enterotoxin (LT). For this purpose, two commercial kits that *E. coli* ST EIA was used to determine of ST and VET-RPLA was used to determine of LT. A total of 695 *E. coli* strains were isolated and identified from 2300 fecal samples. Out of these isolates tested for the presence of ST and LT enterotoxin, 17 isolates (2.4%) were found positive for ST and 13 isolates (1.9%) for LT. *E. coli* strains isolated from turkey, pigeon, gull and ostrich were found to be negative for ST and LT. As a result, with this study, the enterotoxigenic properties of *E. coli* strains isolated from animal species such as quail, gull, pigeon and ostrich were examined for the first time in Turkey.

Key words: Enterotoxigenic *E. coli*, isolation, ELISA, latex agglutination, LT, ST, animal

INTRODUCTION

Escherichia coli is a major component of the normal intestinal flora of animals. This bacterium belongs to many different serotype and can be isolated from the feces of both healthy and diseased animals. Whereas most of strains of *E. coli* are not pathogenic, some of them have acquired genes that can impart virulence. The pathogenic *E. coli* strains have been associated with gastroenteric diseases, such as diarrhea and hemorrhagic colitis. Pathogenic *E. coli* strains cause significant loss of neonatal animals (DebRoy and Maddox, 2001). Certain *E. coli* strains isolated from animals may also cause disease in humans via food borne transmission (Güler *et al*., 2008).

Enterotoxigenic severe diarrhea of two distinct *Escherichia coli* (ETEC) can cause in humans and animals by production types of enterotoxin, a heat-Labile enterotoxin (LT) with two subtypes (LTI and LTII) and a family of heat-Stable enterotoxin (ST). These enterotoxin reversibly alter normal intestinal homeostasis, causing intestinal secretion and diarrhea. LT is a high-molecular-mass toxin (85 kDa) functionally and structurally related to *Vibrio cholerae* toxin. ST is low-molecular-mass toxins that retain toxic activity after incubation at 100°C for 30 min, whereas LT loses activity under these conditions (Dubreuil, 1997). For detection of these toxins, Enzyme Immunoassay (EIA), Passive haemagglutination (PA), various latex agglutination tests, ligated small intestinal loop assays, Infant Mause Assay (IMA), cell culture cytotoxicity assays, DNA hybridization techniques, immunoblot and different PCR methods may be used (Carroll *et al*., 1990; Blanco *et al*., 1997; Orden *et al*., 2002; Guler *et al*., 2008; Hossain *et al*., 2008; Rajkhowa *et al*., 2009). ELISA and latex agglutination tests are known to be simple to perform, easy to interpret and highly sensitive to detect ST and LT types of *E. coli* strains.

This study was carried out to investigate the heat-stabile enterotoxin by using *E. coli* ST EIA test kit and heat-labile enterotoxin by using VET-RPLA test kit in *E. coli* strains isolated from fecal samples of sheep, cow, dog, quail, cattle, ostrich, lamb, gull, chicken, Turkey, cat and pigeon in Van and around in Turkey.

MATERIALS AND METHODS

Isolation of *E. coli* strains: Fecal samples from all animals were collected using sterile swabs and were brought to the laboratory for further processing. Fecal samples were directly inoculated on MacConkey agar (Oxoid, UK) plates. After overnight incubation at 37°C, *E. coli* like colonies were picked up and subcultured on eosin methylene blue agar (Oxoid, UK) plates to observe the

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characteristic metallic sheen. The well separated colonies were picked up on nutrient agar slants as pure culture and subjected to standard morphological and biochemical tests to ascertain their identity as *E. coli* (Holt *et al*., 1994).

Detection of stabile toxin: For this purpose competitive EIA test kit (*E. coli* ST EIA, Oxoid, TD700) was used. *E. coli* strains inoculated 2 mL of sterile CA-YE broth and incubated the culture, with continuous and vigorous shaking, at 37°C for 18-24 h. After incubation, cultures were centrifuged at 900 g for 30 min at 4°C and used the supernatant as the test sample. Working reagents (antibody-enzyme conjugate, wash buffer and substrate solution) were prepared according to manufacturers instruction manual. Each strip of 16 wells was accommodated 13 specimens in addition to positive, negative and culture medium controls. The plastic sealer was removed, then placed the strip (s) in the holding frame and just before use, discarded the well contents. And then wash buffer was added to each of the wells and again discarded the contents. Two hundred microliters of negative control, positive control and sterile culture medium was added in first three wells of each strip. Using fresh tips each time, added 200 μL of each test sample (culture supernatant) to the remaining wells as required. Ten microliter of the diluted antibody-enzyme conjugate was added to every well. To mix the contents of each well, a micro mixer was used by rotating the plate.

Plates were covered and incubated at room temperature for 90 min. After the incubation period, the contents from the wells were aspirated. Two hundred microliter of diluted wash buffer was added to each well and mixed for 30 sec and discarded the liquid into disinfectant. This washing procedure was repeated a further 4 times. One hundred microliter of freshly prepared substrate solution was added to every well used, including the three control wells and mixed for a few seconds. The plate was leaved at room temperature, avoiding strong sunlight, for 30 min to allow the color to develop. Then carefully was added 100 μL of the stopping reagent to each well. The test had been performed correctly if the positive control well was colorless, the negative control well showed a yellow color and the culture medium control showed a yellow color and had a similar optical density as that of the negative control. Then the color intensity produced by each specimen with the three controls was compared (by eye). A specimen with a color intensity corresponding to that of the positive control was regarded as a definite positive. Similarly, a specimen with a color intensity corresponding to that of the negative control was regarded as a definite negative. Specimens which show an ambiguous or uninterpretable result were read by a microlitre plate reader at a wavelength of 490 mm, using the following procedure. Optical Density (OD) of all the wells including positive, negative and culture medium controls were measured and determined the adjusted OD of each of the test samples using the following formula: Adjusted OD = specimen OD divided by negative control OD minus positive control OD. A positive result for ST was given by an adjusted OD value of <0.5. A negative result for ST was given by an adjusted OD of >0.5. For a specimen was regarded as ST positive, it must had an OD which was lower than that of the culture medium control (Oxoid Manual, 2006).

Detection of labile toxin: For this purpose, reversed passive latex agglutination test kit (VET-RPLA, Oxoid, TD0920A) was used. *E. coli* strains were inoculated into 2 mL of sterile Mundell's medium and incubated at 30°C for 24 h with shaking. To the overnight broth culture polymyxin B was added to a concentration of 10.000 units mL^{-1} and incubated at 37°C for 4 h. After incubation, cultures were centrifuged at 900 g for 20 min at 4°C and the supernatant used as test samples. Test was performed in microtitre plate (V-well). Latex and control reagent were diluted and prepared to use. For each sample 2 rows were used. Twenty five microliter of diluents' was dispensed in each well of the 2 rows except for the first well in each row. And then 25 μL of test sample was added to the first and second well of each row. Starting at the second well of each row, 25 μL liquid was picked up and performed doubling dilutions along each of the 2 rows and stopped at the 7th well to leave the last well containing diluents only. Then, 25 μL of sensitized latex was added to each well of the first row. Twenty five microliter of latex control was added to each well of the second row and mixed the contents of each well, rotate the plate by micro mixer. Plate was covered with a lid to avoid evaporation and leaved at room temperature, for 20-24 h. After incubation were examined each well in each row for agglutination, against a black background. The agglutination pattern was judged by comparison with control latex and positive control (Oxoid Manual, 2006).

RESULTS

A total of 695 *E. coli* strains were isolated and identified from 2300 fecal samples taken from 12 different animal species in the present study (Table 1). Out of these isolates were tested for the presence of ST and LT enterotoxin, 17 isolates (2.4%) were found positive for ST and 13 isolates (1.9%) for LT. The distribution of toxin types by animal species was given in Table 2.

Table 1: The distribution of isolated *E. coli* strains by animal species

Animal species	No. of fecal samples examined	No. of *E. coli* strains isolated (%)
Gull	335	56 (16.7)
Bovine	255	60 (23.5)
Chicken	235	64 (27.2)
Lamb	205	63 (30.7)
Sheep	195	60 (30.8)
Cow	185	55 (29.7)
Pigeon	185	58 (31.4)
Turkey	175	58 (33.1)
Cat	165	56 (33.9)
Ostrich	135	55 (40.7)
Quail	125	54 (43.2)
Dog	105	56 (53.3)
Total	2300	695 (30.2)

Table 2: The distribution of stabile and labile toxin types by animal species

	Enterotoxin type					
Animal species (n)	ST	%	LT	%	Total	%
Cow (55)	7	12.7	1	1.8	8	14.5
Sheep (60)	2	3.3	2	3.3	4	6.7
Lamb (63)	2	3.2	2	3.2	4	6.3
Quail (54)	-	-	2	3.7	2	3.7
Dog (56)	1	1.8	2	3.6	3	5.4
Cat (56)	2	3.6	1	1.8	3	5.4
Bovine (60)	2	3.3	1	1.7	3	5.0
Chicken (64)	1	1.6	2	3.1	3	4.7
Turkey (58)	-	-	-	-	-	0.0
Pigeon (58)	-	-	-	-	-	0.0
Gull (56)	-	-	-	-	-	0.0
Ostrich (55)	-	-	-	-	-	0.0
Total (695)	17	2.4	13	1.9	30	4.3

DISCUSSION

Cell culture, PCR, DNA hybridization, bioassay, ELISA and latex agglutination tests have been used for determination of enterotoxin types in *E. coli* strains. Although, sensitivities of these methods are different from each other, ELISA and latex agglutination tests have widely been used for detecting and typing of ETEC strains in culture and feces (Mills and Tietze, 1984; Blanco *et al*., 1997; Guler *et al*., 2008; Hossain *et al*., 2008).

Significant numbers of studies were performed for determination of enterotoxin types of *E. coli* strains from animal origins. In a study on bovine (Carroll *et al*., 1990), it was reported that 16 *E. coli* strains, which were positive for STIa by DNA probes were positive for ST by competitive EIA, but were found to be negative for LT by latex agglutination. Similar results were reported from Spain in a study performed diarrheic and healthy bovine (Blanco *et al*., 1993b). In the previous study carried out in India (Rajkhowa *et al*., 2009), out of 54 *E. coli* strains isolated from bovine was found to be positive 2 (3.7%) for ST gene and 1 (1.8%) for LT gene by PCR. Similar observations were also reported by Mainil *et al*. (1990) and Shin *et al*. (1994).

In a study carried out in Brazil (Salvadori *et al*., 2003) 8 (3.9%) of 250 *E. coli* strains isolated from diarrheic calves were found to be ST, 17 (8.3%) for LT. Rigobelo *et al*. (2006) reported that high percentage of ST (25.4%) and LT (13.2%) genes were in diarrheic calves in same country. In a similar study was performed in Turkey (Güler *et al*., 2008) 12 (16%) of 75 *E. coli* strains isolated from diarrheic calves were found to be enterotoxigenic, but all 45 isolates from healthy calves were found negative for STa by multiplex PCR. Osek and Winiarczyk (2001) reported that all of 390 *E. coli* strains isolated from healthy calves were negative for ST and LT by PCR. In another study (Mills and Tietze, 1994), 6 (2.4%) of 251 *E. coli* strains of calf origin were determined for ST by ELISA. Wolk *et al*. (1992) reported that 35 (58.3%) of 60 *E. coli* isolated from calves and 4 (66.7%) of 6 isolates of lamb origin were positive for ST by ELISA, but all strains tested by same method were negative for LT. In a study carried out in lambs (Orden *et al*., 2002), 2 (1.4%) of 146 *E. coli* strains were determined ST gene by PCR. Blanco *et al*. (1996) also reported that 2 (1.4%) of 144 *E. coli* strains isolated from diarrheic lambs were positive for LT.

Morato *et al*. (2008) reported that all of 300 *E. coli* strains isolated from cat, 230 healthy and 70 diarrheic, were negative for ST and LT genes by PCR. Similar observations were also reported by other researchers (Abaas *et al*., 1989; Blanco *et al*., 1993a). Masalmeh *et al*. (1990) detected 40 (14.2%) of 281 *E. coli* strains isolated from cat and dogs for ST by DNA probe, 22 (7.8%) for LT by Y1 cell culture. In a study was performed in *E. coli* strains isolated from dog with gastroenteritis 5 (20.8%) of 24 hemolytic strains were detected ST by DNA probe, but all of isolates were found to be negative for LT by Y1 cell culture (Prada *et al*., 1991). Other researchers were reported similar findings (Wasteson *et al*., 1988; Hammermueller *et al*., 1995; Holland *et al*., 1999; Starcic *et al*., 2002).

Vidotto *et al*. (1990) reported that all of 45 *E. coli* strains isolated from chicken were negative for ST by IMA. In a similar study (Blanco *et al*., 1997), only one (0.2%) of 458 *E. coli* strains from septisemic chicken origin was found positive for LT, but all of 167 *E. coli* strains isolated from normal chicken were found negative for ST and LT. Similar findings were announced by Akashi *et al*. (1993). In another study performed septicemic poultry, 24 (6%) of 420 *E. coli* strains isolated from turkey and 6 (7%) of 80 isolates of chicken origin were determined LT by Vero and Y1 cell cultures (Emery *et al*., 1992).

In present study, frequency of ST and LT enterotoxin in the fecal samples of healthy animals was low. Thirty (4.3%) of 695 isolates were found to be positive for ST and LT enterotoxin, 17 isolates (2.4%) for ST and 13 isolates (1.9%) for LT. When these results were evaluated by animal species 7 (12.7%) of 55 *E. coli* from cow origin were found to be positive for ST and 1 (1.8%)

for LT; 2 (3.3%) of 60 sheep isolates for ST and 2 (3.3%) for LT; 2 (3.2%) of 63 lamb isolates for ST and 2 (3.2%) for LT; 2 (3.7%) of 54 quail isolates for LT; 1 (1.8%) of 56 dog isolates for ST and 2 (3.6%) for LT; 2 (3.6%) of 56 cat isolates for ST and 1 (1.8%) for LT; 2 (3.3%) of 60 bovine isolates for ST and 1 (1.7%) for LT; 1 (1.6%) of 64 chicken isolates also found positive for ST and 2 (3.1%) for LT. *E. coli* strains isolated from turkey, pigeon, gull and ostrich were found to be negative for ST and LT.

When the data of previous studies take into consideration, it has showed that variable percentages of ST and LT were seen among domestic animals in *E. coli* strains. It was reported that these toxins were low (Blanco *et al*., 1993b; Shin *et al*., 1994; Rajkhowa *et al*., 2009) or usually found to be negative (Osek and Winiarczyk, 2001; Guler *et al*., 2008; Morato *et al*., 2008) in studies performed in healthy animals. On the contrary, those rates were determined high in studies carried out on diarrheic animals (Hammermueller *et al*., 1995; Salvadori *et al*., 2003; Rigobelo *et al*., 2006). In this study carried out on healthy animals being low percentages of ST and LT and not detecting these toxins in some isolates support these opinions that posed by Guler *et al*. (2008), Morato *et al*. (2008) and Rajkhowa *et al*. (2009).

CONCLUSION

As a result, with this study, heat-stabile and heat-labile enterotoxin types of *E. coli* strains isolated from animal species such as quail, gull, pigeon and ostrich were examined for the first time in Turkey. It was concluded that this research would guide and contribute the studies to be made in this field in Turkey and other places.

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