Occurrence of extended-spectrum cephalosporinase producing *Escherichia coli* in kuroiler birds

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GHOSH, P., A. MAHANTI, I. SAMANTA, S. N. JOARDAR, K. BATABYAL, S. DEY, S. TARAPHDER, D. P. ISORE: Occurrence of extended-spectrum cephalosporinase producing *Escherichia coli* in kuroiler birds. Vet. arhiv 87, 745-757, 2017.

ABSTRACT

The present study was undertaken to detect the incidence of extended-spectrum-cephalosporinase gene possessing-*Escherichia coli*, its co-resistance pattern against other antimicrobials, and the clonal relationship of the isolates in healthy kuroiler birds. A total of 80 cloacal swabs from kuroilers were collected randomly from West Bengal, India. The use of costly antimicrobials (cephalosporins) was not practiced by farmers. *Escherichia coli* was isolated and identified by standard biochemical tests and 16SrRNA-PCR. All the *E. coli* isolates, including controls, were subjected to PCR for detection of bla_{CTX-M}, bla_{TEM}, bla_{SHV}, and bla_{CMY-2} genes. By comparing the RAPD-banding pattern, the phylogenetic relationship among the isolates was established. All the isolates were tested for phenotypical resistance against other antibiotics. In total, 60 isolates were identified as *E. coli* from the kuroilers studied (n = 80). Among them, 12 (20%) isolates possessed one of the studied extended-spectrum cephalosporinase genes. Among the studied genes, bla_{TEM} and bla_{SHV} were detected in 6 (10%) and 12 (20%) *E. coli* isolates, respectively. None of the *E. coli* isolates possessed bla_{CTX-M} and bla_{CMY-2}. In phylogenetic analysis, the strains isolated from same localities with similar genetic profile were grouped into the same cluster. Resistance of extended-spectrum cephalosporinase gene possessing *E. coli* isolates was observed most frequently against ampicillin/cloxacillin, co-trimoxazole, amoxyclav, piperacillin, ceftriaxone, and tetracycline. Kuroiler birds with no cephalosporin usage profile may act as a reservoir of extended-spectrum cephalosporinase gene possessing *E. coli*. This is the first systematic study in kuroilers, to raise the awareness of consumers regarding the possibility of transmission of antimicrobial resistant *E. coli* from them.

Key words: *E. coli*, extended-spectrum cephalosporinase, India, kuroiler

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Introduction

Transmission of zoonotic bacteria from animals or birds to humans is further complicated by the association of antimicrobial resistance. Among different mechanisms selected by the antimicrobial resistant *Escherichia coli* strains, production of cephalosporinase, such as extended-spectrum beta-lactamase (ESBL) and ampC β-lactamases, has been commonly observed over past decades (REICH et al., 2013). The presence of extended-spectrum beta-lactamase (ESBL) producing *E. coli* in humans can confer resistance to a variety of β-lactam and other antibiotics, including penicillins, 2nd, 3rd and 4th-generation cephalosporins and monobactams (e.g. aztreonam), but usually not carbapenems or cephamycins (MARCHAIM et al., 2010). However, ampC β-lactamases provide resistance to penicillins, 2nd and 3rd-generation cephalosporins, including β-lactam/inhibitor combinations, cefamycins (cefoxitin), but usually not to 4th-generation cephalosporins (cefepime, cefquinome) and carbapenems (EFSA PANEL ON BIOLOGICAL HAZARDS, 2011) The economic burden of antimicrobial resistance is significant, due to delayed response to treatment and prolonged stay in hospital (DE KRAKER et al., 2011). There are three classical ESBLs, i.e. TEM (except TEM-1), SHV (except SHV-1 and 2) and CTX-M (EFSA PANEL ON BIOLOGICAL HAZARDS, 2011).

Among food animals that act as reservoirs of ESBL-producing *E. coli*, broilers are considered to be the most potent reservoir (PACHOLEWICZ et al., 2015). Almost all the ESBL genes are carried by mobile genetic elements of *E. coli,* and the genes can spread both clonally and horizontally among different lineages of *E. coli* (GHODOUSI et al., 2015). Earlier studies revealed the similarity between ESBL-producing *E. coli* strains isolated from humans and chicken meat, based on multilocus sequence typing, phylotyping, ESBL genes, plasmid replicons, virulence genes, amplified fragment length polymorphism and pulsed-field gel electrophoresis, which suggests the transmission and colonization possibility of bacteria or their genes in humans (KLUYTMANS et al., 2013).

The possibility for all animals and birds to act as reservoirs of extended-spectrum cephalosporinase-producing *E. coli* and the route of transmission from the reservoirs is still unexplored (EFSA PANEL ON BIOLOGICAL HAZARDS, 2011). Moreover, extended-spectrum cephalosporinase-producing *E. coli* usually do not produce any clinical symptoms in the animals or birds acting as reservoirs. Other than broilers, ducks (MA et al., 2012), turkeys (RANDALL et al., 2011) and wild birds (GUENTHER et al., 2012) have so far been explored as reservoirs of beta lactamase-producing *E. coli*.

In India, 'kuroiler' birds were introduced in the early 1990s by a private farm for farmers, as a new opportunity for better production of eggs and meat. Kuroilers are a dual purpose (egg and meat type) breed of birds, with higher productivity than indigenous breeds. The birds are successfully reared in a backyard system because they can easily scavenge food like indigenous birds (AHUJA et al., 2008). In India, earlier studies conducted

in healthy livestock, such as cattle, pigs and broilers, revealed the existence of ESBL-producing *E. coli* and *Klebsiella pneumoniae* (LALZAMPUIA et al., 2013; LALZAMPUIA et al., 2014; SAMANTA et al., 2015; SAMANTA et al., 2015a; KAR et al., 2015; MANDAKINI et al., 2015). The status of other domestic birds such as kuroilers as reservoirs of extended-spectrum cephalosporinase-producing *E. coli* is still unexplored.

The present study was undertaken to detect the occurrence of extended-spectrum cephalosporinase gene possessing-*E. coli* (ESBL and ampC beta lactamase producers) in healthy kuroiler birds reared in West Bengal, India. The study was also intended to reveal co-resistance patterns against other antibiotics and the clonal relationship of the isolates.

Materials and methods

A total of 80 cloacal swabs from kuroiler birds were collected randomly from different locations of the Darjeeling District, West Bengal, India (Table 1). Samples were collected from apparently healthy birds of both sexes, from 3 months to one year in age. The birds were reared by intensive, semi-intensive or backyard systems. Use of costly antimicrobials (e.g. cephalosporins) for both prevention and therapeutic purposes was not practiced by the farmers. Occasionally the birds, including the sampled birds, were treated with amoxicillin and tetracycline during an outbreak.

Table 1. Locations of sample collection in Darjeeling district, West Bengal, India

District	Block	Village	No. of samples collected
Darjeeling, West Bengal	Bijanbari	Rajbari	28
	Sukhia Pokhari	Mem Kaman Tea Estate	26
	Kurseong	Fatak	14
	Darjeeling	Singamari	12
		Total	80

The cloacal swabs were collected by sterile cotton swab sticks (HiMedia, India) and kept in sterile peptone water (HiMedia, India) for transport. The samples were brought to the laboratory maintaining the cold chain, and were processed within 48 hours.

The cloacal swabs collected in peptone water were inoculated into MacConkey's agar (HiMedia, India) and incubated at 37 °C for overnight. The next day rose pink colonies were picked and transferred into EMB agar (HiMedia, India) and again incubated overnight at 37 °C. The colonies were observed after incubation and a single colony was streaked into nutrient agar (HiMedia, India) slants for further morphological and biochemical confirmation (QUINN et al., 1994).

The morphologically and biochemically verified *E. coli* isolates were subjected to PCR for confirmation, as described by WANG et al. (1996). The positive control,

supplied by the Department of Veterinary Microbiology, Central Agricultural University, Mizoram, India, and sterile distilled water as the negative control were included in the PCR. The amplified product was visualized by a gel documentation system (UVP, UK) after electrophoresis in 2% (W/V) agarose (SRL, India) gel containing ethidium bromide (0.5μg/ml) (SRL, India) (SAMBROOK and RUSSEL, 2001).

All the *E. coli* isolates, including controls, were subjected to PCR for detection of bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{CMY-2} genes, using the primers and cycle conditions described earlier (CAO et al., 2002; WEILL et al., 2004; SMET et al., 2008). The positive control supplied by the Department of Veterinary Microbiology, Central Agricultural University, Mizoram, India, and sterile distilled water as the negative control were included in the PCR. The primers used for amplification of CTX-M, SHV and TEM in the study are specific for CTX-M-9, SHV-12 and TEM-1, respectively.

The molecular characterization of all the extended-spectrum cephalosporinase gene possessing *E. coli* isolates was performed by RAPD-PCR (LIM et al., 2009). All the images captured by the gel documentation system were analyzed using Doc-itLs image analysis software, as per the manufacturer's instruction (UVP, UK). By comparing the differences in the banding pattern, the phylogenetic relationships among the isolates were established. An unrooted phylogenetic tree was made using the neighbour joining method.

All the extended-spectrum cephalosporinase gene possessing *E. coli* isolates were sent for serogrouping to the National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasuli, HP, India.

All the extended-spectrum cephalosporinase gene possessing *E. coli* isolates were tested using amikacin (30 μg), ampicillin/cloxacillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), ceftriaxone (30 μg), gentamicin (10 μg), piperacillin (30 μg), ampicillin/sulbactum (10/10 μg), enrofloxacin (10 μg), imipenem-EDTA (10/75 μg), tetracyclin (30 μg), azithromycin (15 μg), and co-trimoxazole (25 μg) discs (Himedia, India) to study the sensitivity pattern of the isolates by the disc diffusion method (CLSI, 2008). The minimum inhibitory concentration breakpoints were used to interpret the zone diameter for each antibiotic, as mentioned in the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008). Discs with CLSI-recommended antibiotic concentrations were used. However, if not available, a disc (co-trimoxazole) with the concentrations provided by the manufacturer was used. The positive control supplied by the Department of Veterinary Microbiology, Central Agricultural University, Mizoram, India was included in the study.

The occurrence of different extended-spectrum cephalosporinase genes among the *E. coli* strains isolated from kuroilers was compared by chi- square test using SPSS software version 17.0 (SPSS Inc.).

Results

In the present study, a total of 60 isolates were presumptively identified as *E. coli* from the 80 cloacal samples of kuroiler birds examined. All the presumptive *E. coli* isolates showed the characteristic pink coloured colony in MacConkey agar, metallic sheen in EMB agar and the isolates were gram negative rods. The isolates showed the standard results for the biochemical tests conducted such as catalase (+ve), oxidase (-ve), Indole-Methyl Red-Voges Proskauer-Citrate (+ + - -) and Urease (-ve). All the 60 presumptive *E. coli* isolates were positive for 16s rRNA with an amplified product size of 585 bp in PCR.

Among the 60 *E. coli* isolates, 12 (20%) isolates possessed one of the studied extended-spectrum cephalosporinase genes. Among the three *bla* genes studied, bla_{TEM} and bla_{SHV} were detected in 6 (10%) and 12 (20%) *E. coli* isolates, respectively (Table 2). None of the *E. coli* isolates possessed bla_{CTX-M} and bla_{CMY-2} (Table 2). The majority of the extended-spectrum cephalosporinase gene possessing *E. coli* belonged to UT and O126 serogroups (Table 2).

Table 2. Genotype of extended-spectrum cephalosporinase *E. coli* (n = 12) isolated from faecal samples of kuroilers in West Bengal, India

Sample Nº	*E. coli* 16S rRNA PCR	bla_{SHV}	bla_{TEM}	bla_{CTX-M}	bla_{CMY-2}	Serogroup
PEC2	+	+	-	-	-	O126
PEC3	+	+	+	-	-	O126
PEC4	+	+	+	-	-	OR
PEC5	+	+	-	-	-	OUT
PEC7	+	+	-	-	-	OUT
PEC8	+	+	+	-	-	O126
PEC9	+	+	+	-	-	O77
PEC10	+	+	-	-	-	O83
PEC11	+	+	+	-	-	OUT
PEC12	+	+	+	-	-	OUT
PEC13	+	+	-	-	-	OUT
PEC14	+	+	-	-	-	OUT
	n = 12	n = 12	n = 6	n = 0	n = 0	

All 12 extended-spectrum cephalosporinase gene possessing *E. coli* isolates were characterized by RAPD-PCR to determine the genetic diversity. All the isolates were typeable with the RAPD primer, and the amplified fragment size was detected in the range of 150 bp to 2165 bp (calculated by Doc-itLs image analysis software, UVP, UK).The phylogenetic analysis revealed the absence of serogroup specific cluster. The extended -spectrum cephalosporinase-gene possessing *E. coli* strains isolated from same localities

(PEC8/PEC10, PEC9/PEC11/PEC12) were grouped in the same clusters. Moreover, *E. coli* strains with similar extended-spectrum cephalosporinase genes (*bla*$_{SHV}$ possessing PEC7, PEC10, PEC13) and (*bla*$_{TEM}$ and *bla*$_{SHV}$ gene possessing PEC3, PEC9, PEC11, PEC12) were also grouped in the same cluster in the dendogram (Fig. 1).
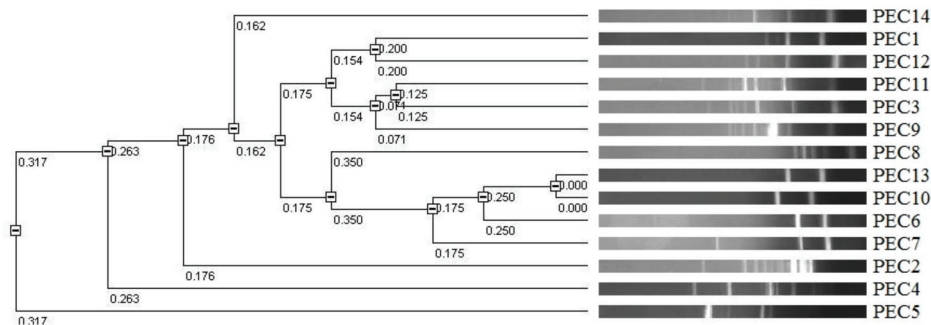

Fig. 1. Phylogenetic analysis of ESBL gene possessing *E. coli* strains isolated from healthy kuroilers in West Bengal (India). The neighbour-joining method was used to summarize the similarity of RAPD-PCR profiles of ESBL gene possessing *E. coli* strains in a dendogram.

Table 3. Phenotypical resistance pattern of extended-spectrum cephalosporinase *E. coli* (n = 12) isolated from faecal samples of kuroilers in West Bengal, India

Antibiotic (Conc. in µg)	No. of isolates Sensitive (%)	No. of isolates Intermediate (%)	No. of isolates Resistance (%)
Amikacin (30 µg)	10 (83.33)	1 (8.33)	1 (8.33)
Amoxycillin/clavulanic acid (20/10 µg)	2 (16.66)	0 (0)	10 (83.33)
Ampicillin/Sulbactum (10/10 µg)	6 (50.00)	4 (33.33)	2 (16.66)
Azithromycin (15 µg)	3 (25)	4 (33.33)	5 (41.66)
Ceftriaxone (30 µg)	2 (16.66)	2 (16.66)	8 (66.66)
Co-Trimoxazole (25 µg)	1 (8.33)	0 (0)	11 (91.66)
Enrofloxacin (10 µg)	9 (75.00)	1 (8.33)	2 (16.66)
Gentamicin (10 µg)	12 (100)	0 (0)	0 (0)
Imipenem/EDTA (10/75 µg)	12 (100)	0 (0)	0 (0)
Piperacilin(30 µg)	2 (16.66)	0 (0)	10 (83.33)
Tetracycline (30 µg)	4 (33.33)	0 (0)	8 (66.66)
Ampicillin/Cloxacilin (10 µg)	0 (0)	0 (0)	12 (100)

The resistance of extended-spectrum cephalosporinase gene possessing *E. coli* isolates was observed most frequently against ampicillin/cloxacillin (100%), co-trimoxazole (91.66%), amoxyclav (83.33%), piperacillin (83.33%), ceftriaxone (66.66%), and tetracycline (66.66%), and less frequently against amikacin (8.33%),

enrofloxacin (16.66%), and ampicillin/sulbactum (16.66%). No resistance was observed against imipenem/EDTA and gentamicin. All the isolates were resistant to three or more antimicrobial agents (Table 3).

Discussion

Escherichia coli harbouring any of the studied extended-spectrum cephalosporinase genes was detected in a few isolates (12/60, 20%) from West Bengal, India. Inclusion of susceptibility testing prior to PCR, for detection of resistance genes, might increase the number of isolates. Among the conventional and semi-automated systems for detection of ESBL-*E. coli*, the double disc test was reported earlier with the highest specificity and positive predictive value of all test methods (WIEGAND et al., 2007). This kind of occurrence study in kuroiler birds has apparently not been undertaken elsewhere in the world for comparison of findings. Prior studies in broilers in West Bengal revealed a higher occurrence rate (29.4%) of ESBL-*E. coli* (SAMANTA et al., 2015a). However, in other states in India, such as in Odisha and in Mizoram, the occurrence of ESBL-*E. coli* in broilers was low (3.9%-6.3%) (LALZAMPUIA et al., 2014; KAR et al., 2015). Comparison of extended-spectrum cephalosporinase producing-*E. coli* occurrence with other studies is difficult due to differences in sample size, the methodology used in the laboratory, and geographical location.

Further, a few *E. coli* isolates (12/60, 20%) from kuroiler birds possessed bla_{SHV} in the present study, although there was no history of third generation cephalosporin intake by the birds. However, the present finding in kuroilers, without dietary intake of cephalosporins, is probably due to the use of other antibiotics. Resistance to other antibiotics (amoxicillin, tetracycline, chloramphenicol) is often linked with the generation of bla_{SHV} carrying beta-lactamse producing isolates, due to the linkage between the responsible genes located in the same mobile genetic element (REICH et al., 2013). Moreover, the majority of *E. coli* isolates from the broiler farm environment (rinse water), and the house fly (*Musca domestica*) isolates from the broiler farms, possessed bla_{SHV} (BLAAK et al., 2014, BLAAK et al., 2015). The contaminated environment may play a major role in transmission of bla_{SHV} gene possessing *E. coli* in the kuroiler birds studied. An earlier study in Japan revealed that contamination of the broiler house with ESBL-producing *E. coli* was a greater contributing factor to the occurrence than dietary intake of antibiotics (HIROI et al., 2012). Environmental compartments, such as soil enriched with contaminated manure, water, air and flies, were detected as vehicles for transmission of extended-spectrum beta-lactamase producing bacteria (BLAAK et al., 2015). Further, 6/60 (10%) *E. coli* isolates were detected to possess bla_{TEM} along with bla_{SHV} in PCR. Similarly, bla_{TEM} and bla_{SHV} possessing *E. coli* isolates were detected from poultry meat samples in Italy (GHODOUSI et al., 2015).

None of the *E. coli* isolates from kuroilers were positive for the presence of the bla_{CTX-M} gene by PCR, which is the most conspicuous finding in the present study. Among the ESBL enzymes, CTX-M was observed as the most prevalent type throughout the world specially in humans (CARATTOLI, 2013). Earlier studies in broilers around the world also identified that the majority of extended-spectrum beta-lactamase producing *E. coli* possessed bla_{CTX-M} due to widespread use of cefotaxime and ceftriaxone, or the high mobilization of the encoding gene (RANDALL et al., 2011; REICH et al., 2013; LALZAMPUIA et al., 2014). In the studied kuroilers, cefotaxime or third generation cephalosporins were not used for therapeutic or preventive purposes due to the high cost. Similarly, in our previous study in Rhode Island Red (RIR) backyard chickens, no ESBL-producing *E. coli* and *Salmonella* spp. were detected due to the lack of antibiotic use (SAMANTA et al., 2014; SAMANTA et al., 2014a). Moreover, it seems that the sulfonamide and trimethoprim groups of antimicrobials were also used neither in the studied kuroilers nor in the surrounding ecosystem, as the sulphonamide resistance genes present in class 1 integron may co-select bla_{CTX-M} (SMET et al., 2008).

CMY-2 is considered to be the most prevalent ampC β-lactamase in *E. coli* and *Salmonella* isolated from food animals throughout the world, which led us to select the bla_{CMY-2} gene in PCR (CORTES et al., 2010). None of the *E. coli* isolates from the kuroilers was positive for the bla_{CMY-2} gene. The lack of therapeutic or preventive use of second or third generation cephalosporins in the studied kuroilers could explain the finding. However earlier, in a Swedish study in broilers, CMY-2 was detected in *E. coli* in the absence of cephalosporin use in the diet, and it was concluded that is was carried over from the parental stock (SVARM, 2010).

The majority of the extended-spectrum cephalosporinase gene possessing *E. coli* isolated from kuroilers belonged to an untypeable (UT) serogroup. ESBL-producing *E. coli* belonging to an untypeable serogroup was isolated earlier from piglets in India (MANDAKINI et al., 2015).

The phylogenetic analysis of extended-spectrum cephalosporinase-gene possessing *E. coli* isolates revealed a clonal relationship between the strains belonging to same place and ESBL-gene profile. This finding revealed the presence of some bacterial clones circulating among the kuroilers and the possibility of their spread into the human food chain (JOHNSON et al., 2007), or to other indigenous birds. Kuroilers kept in a backyard occasionally copulate with indigenous birds, which may act as an additional mode of transmission. ESBL-producing *E. coli* were recently detected in the reproductive tract of broiler breeding roosters, which suggests the possibility of the venereal route of transmission (MEZHOUD et al., 2015).

Multidrug resistance among the extended-spectrum cephalosporinase-gene possessing *E. coli* isolates in the present study was detected against co-trimoxazole,

enrofloxacin, amikacin. The resistance against tetracycline among the isolates of the present study could be linked with possession of the bla_{SHV} gene (REICH et al., 2013). The susceptibility of the extended-spectrum beta-lactamase producing *E. coli* isolates to imipenem was also described in earlier study (GOYANES et al., 2007). Two extended-spectrum cephalosporinase-gene possessing *E. coli* isolates were found to be sensitive against ceftriaxone, which carried both bla_{TEM-1} and bla_{SHV-12}. It is difficult to presume which gene played the dominant role for these two isolates.

The present study revealed for the first time that kuroiler birds, with no high generation cephalosporin use, can act as a reservoir of extended-spectrum cephalosporinase-gene possessing *E. coli*. Detection of bla_{SHV} possessing *E. coli* in kuroilers should make consumers cautious about the use of kuroiler meat and eggs, because bla_{SHV} possessing isolates have been sporadically associated with human infections in the community (VOETS et al., 2013).

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SAŽETAK

Istraživanje je poduzeto da se odredi incidencija bakterije *E. coli* koja posjeduje gene za cefalosporinaze proširenog spektra, a istodobno je rezistentna na druge antimikrobne tvari, te klonski odnos izolata u zdravih kokoši pasmine kuroiler. Ukupno je bilo nasumce prikupljeno 80 obrisaka kloake spomenute pasmine u Zapadnom Bengalu u Indiji. Skupe antimikrobne tvari (cefalosporini) nisu bile rabljene za liječenje. Izolati *E. coli* bili su identificirani standardnim biokemijskim testovima i 16SrRNA-PCR-om. Svi izolati bili su lančanom reakcijom polimerazom (PCR) pretraženi na prisutnost gena bla_{CTX-M}, bla_{TEM}, bla_{SHV} i bla_{CMY-2}. Filogenetska srodnost izolata bila je određena RAPD-om. Otpornost svih izolata bila je fenotipski pretražena i na druge antibiotike. Ukupno je izdvojeno 60 izolata *E. coli* iz 80 kuroiler kokoši. Od toga je u 12 (20 %) bio dokazan jedan od istraživanih gena za cefalosporinaze proširenog spektra. Među istraživanim genima, bla_{TEM} je bio dokazan u 6 (10 %) izolata, a bla_{SHV} u 12 (20 %) izolata *E. coli*. Nijedan od izolata nije posjedovao bla_{CTX-M} i bla_{CMY-2}. Filogenetskom analizom ustanovljeno je da su izolati iz istih lokaliteta sa sličnim genetičkim profilom bili grupirani u istu skupinu. Posjedovanje gena za cefalosporinaze proširenog spektra bilo je najčešće dokazano za antibiotike ampicilin/kloksacilin, kotrimoksazol, amoksiklav, piperacilin, ceftriakson i tetraciklin. Perad pasmine kuroiler kojoj nikad nisu davani cefalosporini može biti rezervoar *E. coli* koja ima gene za cefalosporinaze proširenog spektra. To je prvo sustavno istraživanje u pasmine kuroiler od značenja za svjesnost konzumenata o mogućnosti prijenosa bakterije *E. coli* otporne na antimikrobne tvari.

Ključne riječi: *E. coli*, cefalosporinaze proširenog spektra, Indija, kuroiler