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Detection of enterohaemorrhagic *E. Coli* O157: H7 from beef and chevon sold in and around Puducherry

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Abstract
Food borne illnesses have major social and economic impacts. *Escherichia coli* O157:H7 is one of the bacterial organism cause food borne illness in human beings. This study was conducted to detect the presence of *E. coli* O157: H7 from beef and chevon sold in Puducherry as per the standard procedure. A total of 115 meat samples (beef – 55 and chevon - 60) were collected from different retail meat shops in and around Puducherry. Out of 115 samples, 16 samples (13.91 per cent) were positive for *E. coli* O157: H7 by conventional methods. This isolates were further subjected to PCR. Out of 16 isolates 14 (87.5 per cent) isolates were showing presence of *stx*$_2$ gene. After the PCR confirmation that 14 isolates were subjected to the antibiogram and biofilm production assay. The antibiotic resistance profiling of the *E. coli* O157: H7 isolates does not show high resistance. In biofilm production assay by CRA plate method out of 14 isolates, 8 (57.14 per cent) isolates were positive for biofilm production This can be a major concern as microorganisms can be associated with chronic and recurrent human infections. This study clearly indicates the need for proper handling and processing of meat especially beef. It is also important that at household level proper hygienic measures should be taken to avoid cross contamination.

Keywords: *E. coli* O157: H7, Beef, chevon, *stx*$_2$ gene and biofilm

Introduction
Food borne illness defined as usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food. Worldwide food borne and waterborne diarrheal diseases together kill about 2.2 million people annually and in India around 6 per cent of the population. In major causative agents of these illnesses viruses, bacteria, parasites, toxins, metals and prions, especially the bacteria have become an important group of causative organisms as most of morbidity and mortality from food borne illnesses are related to them [22]. Colonization of these bacteria in human beings can cause a broad spectrum of food borne illnesses such as bacteremia, meningitis, urinary tract infection, septicemia, wound infection, central nervous system disorders and gastrointestinal tract disturbances [11]. The estimated mortality rate for different bacterial food borne illness were like *Salmonella* which caused 31 per cent of food related deaths, followed by *Listeria* (28 per cent), *Campylobacter* (5 per cent), and *Escherichia coli* O157:H7 (3 per cent) [27].
Escherichia coli O157:H7 has been first identified as a human pathogen during two outbreaks in Michigan and Oregon involving undercooked hamburger [25]. Since then, infections have been reported from more than 30 countries in six continents. In India, there is little information available on the prevalence of Shiga Toxin producing *Escherichia coli* (STEC) or Enterohemorrhagic *E.coli* (EHEC). There are no major reports, which have identified it as a significant etiologic agent of diarrhoea for humans in India. The few cytotoxic strains of *E. coli* (O157 and non-O157 serogroups) reported from human patients with diarrhoea in India have been uncharacterized with uncertain origin [30]. There are not many reports of isolation of EHEC from various animal species in India. The important animal reservoir of EHEC especially serotypes O157:H7 appears to be mainly cattle and other ruminants such as sheep and goats are considered significant reservoirs, while pigs, horses, rabbits, dogs and cats and birds (chicken and turkeys) have been rarely found infected[23, 17].
The study was conducted to find out the presence of *E.coli* O157:H7 in meat sold in and around Puducherry.

Materials and Methods

The meat samples included beef and chevon samples. These samples were screened for the presence of the organism. The isolates were identified by biochemical methods and then confirmed by molecular characterization of the isolates. All isolates were subjected to antibiotic resistance profiling.

Sample Collection

The study was carried out on the food samples like Beef - 55 and chevon 60 samples. The meat samples of approximately 50-100 grams were collected from different retail meat shops in and around Puducherry. The details of sample collected including the place, type of sample, number of samples etc are given in table no 1.

Table 1: Details of samples collected

S. No	Source	Beef	Chevon
1	Ariyankuppam	3	2
2	Ariyur	2	2
3	Bahoor	4	2
4	Dharumapuri	-	4
5	Gubaran market	-	-
6	Kathirkamam	-	2
7	Kariamanickkam	1	2
8	Kombakkam	3	2
9	Koodapakkam	2	2
10	Kurumbapet	-	3
11	Lawspet	-	2
12	Madagadipet	1	2
13	Madukarai	2	2
14	Mettupalayam	-	3
15	Moollakadai	-	3
16	Muthialpet	2	2
17	Muthuraiyarpallayam	-	2
18	Nellithope Market	-	2
19	Oulgarpet	6	2
20	Pitchaveeranpet	7	3
21	Pondy Town Market	2	2
22	Sultanpet	8	3
23	Thavallakuppam	-	2
24	Thirukanoor	3	2
25	Vaanoor	2	1
26	Vazhudhavur	3	2
27	Villianur	4	4
	Total	55	60

Processing of samples

The collected samples were processed in the Biosafety level–II laboratory facility available in the Department of Veterinary Public Health and Epidemiology, Rajiv Gandhi institute of Veterinary Education and Research, Kurumbapet, Puducherry. Twenty five gram of meat samples were into a stomacher bag and homogenized. The samples remaining after the tests and negative samples were discarded as per standard methods including proper decontamination.

Isolation of EHEC

Isolation and identification of EHEC was carried out by pre-enrichment and selective enrichment followed by selective plating as described by Meng *et al*. [19] For further confirmation, the isolates were plated onto 4-methylumbelliferyl-beta-D-glucuronide (MUG EC O157) agar as described by Fujisawa *et al*. [10]

Pre enrichment

For the pre enrichment, trypticase soya broth supplemented with 20 mg/l Novobiocin (Hi-media) was used. This enrichment procedure was to help in increasing the number of organisms from meat and human stool samples. Twenty five gram of processed meat and sea food (fish and crab) sample was taken for pre enrichment in 225 ml of broth. The mixture was incubated at 37 ^{0}C for 24 hours.

Selective enrichment

The selective enrichment was carried out in *E. coli O157: H7* selective broth. From the pre-enriched homogenized broth culture, 0.1 ml was transferred to 10 ml of selective broth and it was incubated at 37 ^{0}C for 24 hours. The selective enrichment allowed the selective growth of EHEC from samples.

Selective plating

For selective plating, Cefixime Tellurite- Sorbitol Mac Conkey agar (CT- SMAC) (Hi-media) was used. A loopfull of the inoculums of *E. coli* O157:H7 from selective broth was streaked onto selective CT- SMAC agar plates. The plates were incubated at 37 ^{0}C for 48 hours. The positive colonies appeared colourless. For further confirmation, suspected colonies on CT- SMAC agar plates (Non-sorbitol fermenting colourless colonies and neutral grey colonies with smoky centers) were transferred on to MUG EC O157 agar plates and it was incubated at 37 ^{0}C for 24 to 48 hours. After the incubation, colonies were examined under a UV lamp. Light blue fluorescence showed by the colony indicated beta-galactosidase positive and thus confirming that it may be EHEC.

Characterization and identification of isolates:

The suspected colonies of EHEC were subjected to various tests and confirmed based on the biochemical characteristics. The individual colonies of EHEC from CT- SMAC agar were transferred to TSB and incubated at 37 ^{0}C for 24 hours. Primary Identification Tests like Gram's Staining, Catalase Test (Slide test), Oxidase Test and Motility Test were performed. Secondary Identification Tests like Indole production, Methyl Red (MR) Reaction, VogesProskauer (VP) Reaction, Citrate Utilization Test, Urease Activity, Gelatin Hydrolysis/ Liquefactions and Carbohydrate Utilization Test were preceded as per the standard procedures. The characterization/biochemical reaction and carbohydrate utilization pattern of *E. coli* O157:H7 were given in table no. 2.

Table 2: Identification tests for enterohaemorragic *E. coli* O157:H7

S.no	Identification tests	Characterisation/Reactions
1	Gram's staining	Gram negative & Rods
2	Catalase	Positive
3	Oxidase	Negative
4	Motility	Positive
5	Indole production	Positive
6	Methyl red	Positive
7	Voges Proskauer	Negative
8	Citrate utilization	Negative

9	Triple Sugar Iron agar reaction	Positive for both H_2S and gas production
10	Urease test	Negative
11	Sodium Chloride concentration test (0, 3, 6, 8 and 10 per cent)	Negative
12	Lysine decarboxylase	Positive
	Sugars	**Acid from carbohydrate**
13	D-Glucose	Positive
14	Lactose	Positive
15	Sucrose	Positive
16	Sorbitol	Negative
17	D-Mannitol	Positive
18	Inositol	Negative
19	Maltose	Positive
20	Cellobiose	Negative
21	Maltose	Positive
22	Cellobiose	Negative

Maintainance of Isolates

A loopful of the isolate was added to the sterile Dimethyl Sulfoxide (DMSO)-glycerol broth vials each containing around 2 ml of broth and mixed well in the vortex mixer. The vials were then labelled and stored at -20^{0}C. Each isolates were stored in duplicates. The isolates were revived once in three months. The vials stored at -20^{0}C was thawed and streaked on to Nutrient agar and incubated at 37^{0}C overnight. The isolated organism was also streaked onto MH slants in screw- capped test tubes and incubated at 37^{0}C for 24 hours. Once the growth was obtained the slants were stored at 4^{0}C.

Molecular confirmation of the isolates

After the isolation and biochemical characterization, *E. coli* O157:H7 isolates were processed for further confirmation by detection of *stx*$_2$ gene in *E. coli* O157:H7 by Polymerase Chain Reaction (PCR).

Extraction of template DNA

The DNA from *E. coli* O157:H7 isolates were extracted using Boil cell method. A single pure colony of bacterial culture was inoculated into five millilitre of Nutrient broth and incubated at 37^{0}C for 18 hrs. One point five millilitres of this broth culture was transferred to an eppendorf tube and centrifuged at 3000 rpm for 10 minutes. The pellet was washed twice in PBS and then it was resuspended in 100 µl of triple distilled water. This was boiled for 10 minutes and immediately chilled at -20^{0}C for 30 minutes. The sample was then thawed and centrifuged at 3000 rpm for 5 minutes. The supernatant which contained template DNA was stored at -20^{0}C till use. Five micro litres of the supernatant was used as template DNA for PCR. Bacterial culture lysate similarly prepared from the reference strain was used as template DNA for positive control in PCR.

Polymerase Chain Reaction (PCR) for the detection of *E. coli* O157:H7

The following sets of primers were used for the detection of virulent *stx*$_2$ gene of *E.* coli O157:H7 [23] as shown in table no.3 and PCR reaction mixture for *E.* coli O157:H7 was given in table no. 4.

Table 3: Details of primers used

S.No	Organism	Specificity	Primer	Primer sequence (5'-3')	Size
1	*E.* coli O157:H7	nt 603-857 of A subunit coding region of *stx*$_2$	*Stx*$_2$	Forward 5'GGCACTGTCTGAAACTGCTCC3' Reverse 3'TCGCCAGTTATCTGACATTCTG5'	255bp

Table 4: PCR reaction mixture for *E. coli* O157:H7

S. No	Reaction mixtures		Volume (µl)
1	Template DNA		3
2	Primers	Forward	1
		Reverse	1
3	Master mix		10
4	Triple distilled water		5
	Total		20 µl

The 20 µl reaction mixtures were prepared in 0.2 ml thin PCR tubes. The PCR amplification were carried out in an automated thermal cycler (Eppendroff mastercycler, Germany) using protocol in table no. 5.

Table 5: The PCR protocol

S. No	STEPS	*stx*$_2$ *Gene*
1	Primary Denaturation	94°C for 5 minutes
2	Denaturation	94°C for 1 minutes
3	Annealing	60°C for 1 minutes
4	Extension	72°C for 2 minutes
5	Final Extension	72°C for 10 minutes
6	No.of Cycles	35

Submarine agarose gel electrophorsis

The PCR products were analyzed by submarine agarose gel electrophoresis. Agarose (1.5 per cent) was dissolved in TAE buffer (1X) by heating. When the mixture was cooled around 50^{0}C, ethidium bromide was added to a final concentration of 0.5 µg/ml. Agarose was then poured into clean, dry, gel platform setup provided by Tarsons and the comb was kept in proper position. Once gel was set, the comb was removed gently and the tray containing the gel was placed in the buffer tank. Buffer (TAE 1X) was poured till the gel was completely submerged. PCR product (10µl) was mixed with loading buffer (1µl of loading buffer per 5µl of the PCR product) and the samples were loaded into the wells. Then 100 bp DNA markers and positive and negative controls were loaded into separate wells. Electrophoresis was carried out at 5V/cm for one hour. The gel was visualized under UV transilluminator and the images were documented in a gel documentation system (Gel Doc It. Images System, UVP).

Antibiogram

Antibiotic susceptibility tests were performed for *E.* coli O157:H7 according to the National Committee for Clinical Laboratory Standards [21]. Premeasured antimicrobial discs

were applied to the surface of Mueller-Hinton agar plates, previously seeded with each strain and commonly used antibiotics were used in this study.

Preparation of Mac Farland Standard

The turbidity standard solution was prepared by adding 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml of 0.36 N H_2SO_4 (one per cent w/v). This solution is equal to half the density of No.1 Mac Farland standard solution. This solution was taken into the glass tube, sealed tightly and kept in the dark, at room temperature for further use. The tube was vigorously agitated just before each use.

Preparation and standardization of inoculum

Three to four isolated colonies were selected from a pure culture and transferred into a sterile nutrient broth and incubated at 37^0C, overnight. The turbidity of culture was adjusted using solution having half the density of Mac Farland standard No.1. When the broth culture was found to be more turbid, it was diluted with nutrient broth and when the turbidity was found to be less, the culture was incubated for more time to achieve the required turbidity.

Inoculation

The swab was dipped into standardized inoculum and excess inoculum was removed from the swab by rotating it several times with a firm pressure on the inside wall of the test tube, above the fluid level. The sterile Mueller Hinton agar (Hi-Media) plates were inoculated by swabbing over its entire surface, within 15 min. after adjusting the density of inoculum. The swabbing procedure was repeated two more times, rotating the plates approximately 60^0 at each time, so as to ensure an even distribution of inoculums. The inoculums were allowed to dry for 15 min.

Application of antibiotic discs

The inoculated plate was left for not more than 15 min. at room temperature to absorb any excess surface moisture before applying the drug impregnated discs. The discs were applied to the surface of the inoculated agar with a sterile forceps. With the tip of the forceps, each disc was gently pressed down to ensure complete contact with the agar surface. During the application of discs care was taken not to place it closer than 15mm from the edge of the plate and the distance between the centre's of two such discs was not less than 24mm. The inoculated plate was inverted and incubated at 37^0C for 18 hours after the application of the discs. In this study, 15 locally available antibiotic discs were tested against both *E. coli* and *Vibrio parahaemolyticus* isolates. The isolates were tested against Ampicillin (10 mcg), Amoxyclav (30 mcg), Amikacin (30 mcg), Azithromycin (30 mcg), Chloramphenicol (30 mcg), Cotrimazole (10 mcg), Ciprofloxacin(30 mcg), Cefotaxime (30 mcg), Gentamicin (30 mcg), Metronidazole (50 mcg), Nalidixic acid (30 mcg), Penicillin-G (10 units) Sulphamethiozole (300 mcg), Trimethoprim (25 mcg), Tetracyclin (30 mcg), Polymixcin B (300 units) antibiotic discs.

Reading and Interpretation

At the end of the incubation period, the plates were examined and the diameter of the zones of complete inhibition was measured to the nearest whole millimeter with a scale held on the back of the Petri plate, which was illuminated with a reflected light. The zone of inhibition of each disc was measured in three different directions keeping the midpoint of the disc as the centre of the zone. The mean of the measurement of inhibition was used for the interpretation of the results. The interpretation of the result was made by comparing diameter of the zone of inhibition with standard zone of inhibition chart provided by the disc manufacturer. The isolates were grouped as sensitive, intermediary sensitive and resistant, against each antibiotic (CLSI, 2010).

Bioflim production assay

Bioflim production in terms of slime production by isolates was determined by cultivation on Congo Red Agar (CRA) plates [9, 5]. A loop full of isolate was streaked onto CRA plates. The plates were incubated at 37°C for 24 hours followed by storage at room temperature for 48 hours. The production of rough black colonies by bacterial cultures indicated the ability of the isolates for bioflim production.

Results and Discussion

Isolation of *E. coli* O157:H7

All the samples collected were subjected to isolation of *E.* coliO157:H7 by conventional culture technique. Species level differentiation was carried out by using carbohydrate utilisation test.

Meat (Chevon and Beef) samples

Out of the 115 meat samples (55 - beef and 60 - chevon) screened, 16 samples (14 samples from beef and 2 samples from chevon samples) were having colonies with characteristics of *E. coli* O157:H7 in SMAC agar. The details of suspected *E.coli* O157:H7 isolates from the meat are shown in the table 6. The growth of *E. coli* O157:H7 suspected isolates in different media are shown in plate no. 1 to 3.All the isolates obtained from meat were subjected to various biochemical tests for further confirmation.

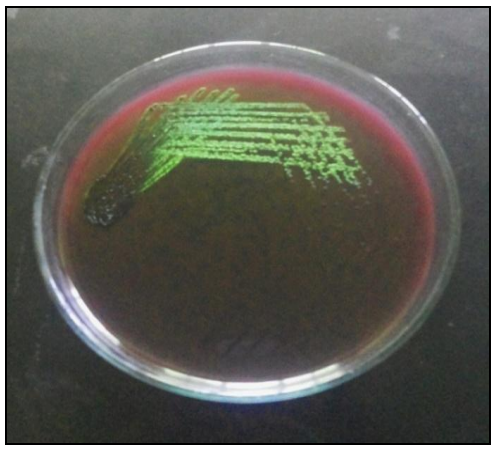

Plate 1: *E. coli* O157:H7 in EMB agar

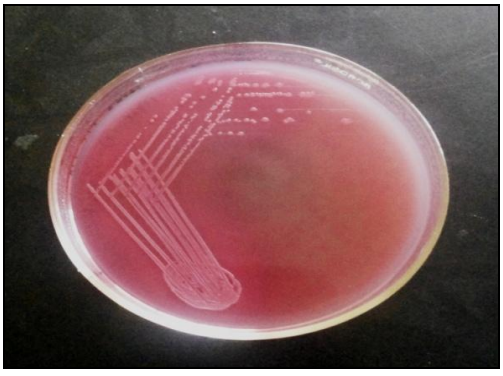

Plate 2: *E. coli* O157:H7 in MacConkey's agar

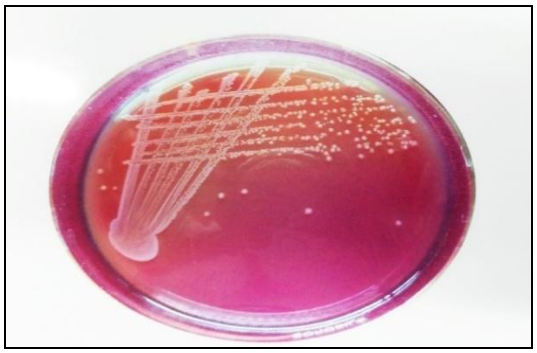

Plate 3: *E. coli* O157:H7 in SMAC agar

Table 6: Details of *E. coli* O157:H7 from meat samples

Samples	Bahour	Kurumbapet	Oulgaret	Pitchaveerampet	Sulthanpet	Vannoor	No. of Positive samples	Per cent
Beef	2	0	2	6	2	2	14/55	25.46
Chevon	0	0	0	1	1	0	2/60	3.33
Total							16/115	13.91

Biochemical tests for *E. coli* O157:H7

When subjected to biochemical tests, it was found that all these isolates were positive for catalase test, motility, indole production, MR, triple sugar iron agar reaction and Lysine decarboxylase. These isolates were negative for oxidase test, VP, citrate utilisation, urease and sodium chloride tolerance test.

The results of these tests clearly indicated that they belonged to the category of enterohaemorragic *E. coli*. These isolates were then subjected to carbohydrate utilisation tests for identification of species. The results suggested that all the 16 isolates were showing reaction similar to that of *E. coli* O157:H7 as shown in tables 2. The details about the biochemical characterization of the 16 isolates are shown in table 7.

The results from the study revealed an overall prevalence of *E. coli* O157:H7 as 13.91 per cent (16/115) in meat collected from different meat retail shops in and around Puducherry. The occurrence of *E. coli* O157:H7 in beef and chevon was found to be 25.46 per cent (14/55) and 3.33 per cent (2/60), respectively. The prevalence observed in the present study is lower when compared to the work done in Malaysia by Radu *et al*. [24] in which they found that 48 per cent of beef samples had the presence of *E. coli* O157:H7. Kumar *et al*. [14] found 100 per cent of the beef samples positive for *E. coli* O157:H7 in a study conducted in Mangalore, India. The USDA [28] reported that the FSIS identified more than 75 per cent of the ground beef samples were positive for the presence of *E. coli* O157:H7. Grant *et al*. [12] reported the prevalence of non-O157 EHEC in raw beef as 2.4 per cent to 49.6 per cent in Canada and in United States it ranged from 5.7 per cent to 26.2 per cent. These suggest that foods, particularly beef may be an important source of *E. coli* O157:H7 infections. In the present study also more occurrences were observed in beef. This may be due to the fact that the gastro intestinal tract of the cattle is the most important predilection site of the organism [17].

The occurrence of *E. coli* O157:H7 of the present study was comparatively higher than a study done by Doyle and Schoeni [6]. They got a prevalence of 3.7 per cent (6/164) from beef and 2.0 per cent (4/205) from lamb meat samples. Almost similar result was found in other studies conducted by Vinothkumar *et al*. [29] in Puducherry, India who observed 22 per cent prevalence in beef samples. Momtaz and Jamshidi [20] in Iran found 21.23 per cent of the beef samples were positive for *E. coli* O157:H7.

These findings suggests that cross-contamination of carcasses may be occurring in retail meat shops and due to higher prevalence in animals. Cattle act as a reservoir host for EHEC O157:H7 resulting in higher food contamination. Mainly in the beef retail shops beef carcasses were not hoisted and kept on the floor for dressing. During handling of carcass the intestinal contents were spilled out and resulted in cross contamination. The amount of water used for cleaning was also less than the standard recommendation. Cutting surfaces and knife were not sanitatized properly. All of these might have led to high prevalence of *E. coli* O157:H7 among the beef samples in the present study.

Table 7: Biochemical characteristics of *E. coli* O157:H7 isolates from meat samples

S.no	Biochemical tests and Sugars	Beef														Chevon			
1	Characterization & Isolates no	1	2	3	10	12	16	19	20	22	25	28	30	31	32	15	18	20	22
2	Shape	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
3	Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	Indole production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	Methyl red	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	Voges proskauer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	Citrate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	TSI	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	Urease test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	Sodium chloride tolerance test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	Lysine decorboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

15	Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	D-mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

R: Rod shaped**, +:** Positive**, -:** Negative

Detection of virulent *stx₂* gene of *E. coli* O157:H7 by PCR

After the biochemical characterization and identification, 16 suspected isolates of *E. coli* O157:H7 were screened by Polymerase Chain Reaction (PCR) for the presence of *stx₂* gene. In that 14 (87.5 per cent) isolates were showing presence of *stx₂* gene (Fig. 1).

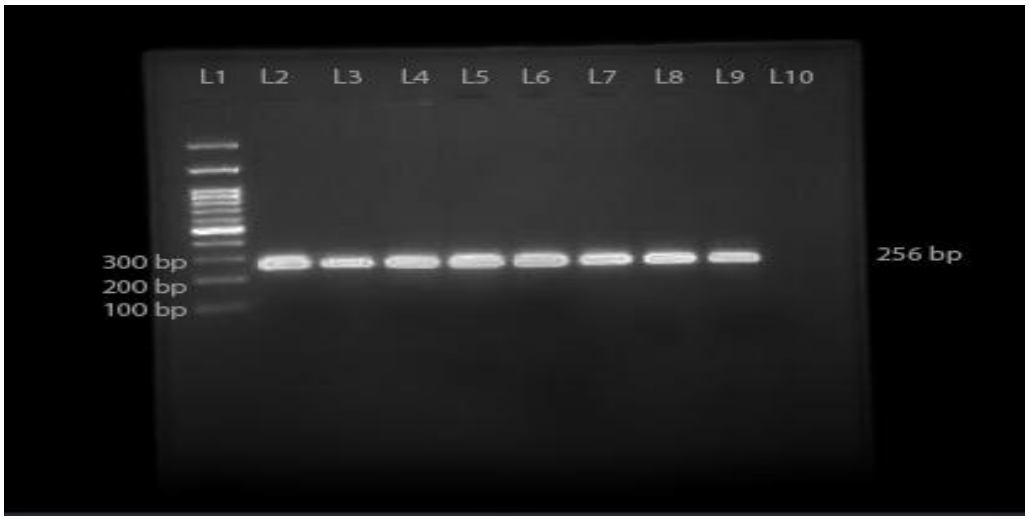

Fig 1: PCR profile of *E. coli* O157:H7 isolates for *stx₂* gene **L1-** 100bp molecular marker, **L2 to L8-** *stx2* gene from positive samples, **L9-** positive control, **L10-** negative control

In the present study, presence of *stx₂* virulence gene was assessed in all the EHEC isolates obtained. Out of 16 suspected isolates obtained, 14 (87.5 per cent) were found to be positive for the presence of the *stx₂* virulence genes by amplification of 255 bp products. Wani *et al*. [31] reported the isolation and characterization of EHEC serogroups associated with diarrhoea in calves and lambs in India. They subjected 130 bovine and 15 ovine strains to PCR for detection of *stx₂* genes and they detected in 9.73 per cent of samples from calves and 6 per cent of samples from lambs studied. Kumar *et al*. [18] revealed PCR was having higher sensitivity than hybridization technique and was used to detect *stx₁* and *stx₂* genes to characterize EHEC strains from seafood and beef. They found shiga toxin genes in 4 strains from seafood and 6 strains from beef in a study conducted in Mangalore. Hazarika *et al*. [14] obtained 27 (24.10 per cent) isolates of *E. coli* from 112 beef samples. When these isolates were subjected to PCR, they found that 77.78 per cent of isolates showed the presence of *stx₂* gene. Baruah *et al*. [1] observed the presence of *stx₂* gene among 17(11.81 per cent) isolates, of that 6 were from piglets, two from calves, three from yaks, two from buffalo calves, and one from goat, one from poultry and two from humans. Kiranmayi *et al.* [17] screened 58 mutton samples and 16 isolates were positive for *stx₂* genes and 16 were positive for *hlyA* gene and 8 were positive for both *stx₁* and *stx₂*. Haugum *et al*. [13] screened a total of 138 patients for EHEC infection by PCR and found that 128 strains of *E. coli* contained the *stx₁* and/or *stx₂* gene. A combination of *stx₁* and *stx₂* was found in 21 (15.2 per cent) strains, while *stx₁* was found in 57 (41.3 per cent) strains and *stx₂* in 50 (36.2 per cent) strains. Bonardi *et al*. [2] found majority of *E. coli* O157 possessed the *stx₂* gene (85.7 per cent) alone and two *E. coli* O157 (14.3 per cent) harbored both *stx₁* and *stx₂* genes.

In the present study out of 16 suspected EHEC O157:H7 isolates, 14 showed the presence of *stx₂* gene and can be confirmed as *E. coli* O157:H7. Based on the results, detection of *stx₂* gene based PCR can be used as an effective tool for confirmation of the isolates.

Antibiogram of the *E. coli* O157:H7 isolates from meat samples

All the 14 isolates of *E. coli* O157:H7 from the meat samples were subjected to the Antibiotic Sensitivity Test (ABST) by standard disc diffusion method with 16 commonly used antibiotics. In that, all isolates showed different resistant patterns against the antibiotics. The detailed antibiogram of the isolates are summarized in table 8 and details about the per cent wise antibiogram profile of isolates from the different samples were shown in figure no.2 and 3. A total of 14 isolates of *E. coli* O157:H7 were almost sensitive to all the antibiotics used in the test.

Escherichia coli O157:H7 isolates from the beef samples showed 100 per cent of sensitivity to ciprofloxacin followed by amipicillin and amikacin at 45.45 per cent, amoxyclav, azithromycin, cotrimazole, cefotaxime, gentamicin, sulphamethazole and tetracyclin at 63.63 per cent, chloramphenicol and metronidazole at 54.54 per cent, cefazolin, moxifloxacin, nalidicxic acid and trimethoprim at 72.72 per cent.

Escherichia coli O157:H7 isolates from the chevon samples showed 100 per cent of sensitivity to amoxyclav, amikacin,cotrimazole, ciprofloxacin, cefotaxime, gentamicin, moxifloxacin and nalidixic acid. Fifty per cent of the isolates were resistant to antibiotics like azithromycin, cholramphenicol, cefazolin, metronidazole and sulphamethazole and remaining 50 per cent were sensitive to

these antibiotics. Isolates showed sensitivity to amipicillin (50 per cent) and resistance to tetracyclin (50 per cent).

Table 8: Antibiogram of *E. coli* O157:H7 isolates

Samples	**Beef**												**Chevon**	
Antibiotics / Isolates no	1	2	3	10	12	16	19	20	28	30	31	32	Ch15	Ch23
Amipicillin	R	S	S	R	S	R	I	S	I	S	R	S	S	I
Amoxyclav	R	S	S	I	S	S	S	S	S	R	I	S	S	S
Amikacin	S	S	I	R	S	I	R	S	S	R	R	S	S	S
Azithromycin	S	I	S	I	S	R	S	S	S	S	R	R	R	S
Chloramphenicol	R	S	I	R	S	R	R	S	S	S	S	S	S	R
Cotrimazole	S	S	I	S	S	I	S	R	S	I	S	R	S	S
Ciprofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefotaxime	S	S	I	R	S	S	I	S	S	R	S	S	S	S
Cefazolin	S	S	S	I	S	S	R	S	S	I	S	S	S	R
Gentamicin	S	R	S	S	S	S	S	I	S	S	R	S	S	S
Metronidazole	S	S	I	S	S	I	S	S	R	I	I	R	R	S
Moxifloxacin	S	S	R	S	S	R	S	R	S	S	S	S	S	S
Nalidixic acid	S	S	S	S	R	R	S	R	S	S	S	S	S	S
Sulphamethiozole	R	S	S	R	S	S	R	S	S	R	S	R	R	S
Trimethoprim	S	S	S	S	R	R	S	R	S	S	S	I	S	S
Tetracyline	S	R	S	S	R	S	R	S	S	R	S	R	R	I

S: Sensitivity, **R:** Resistant, **I:** Intermediate

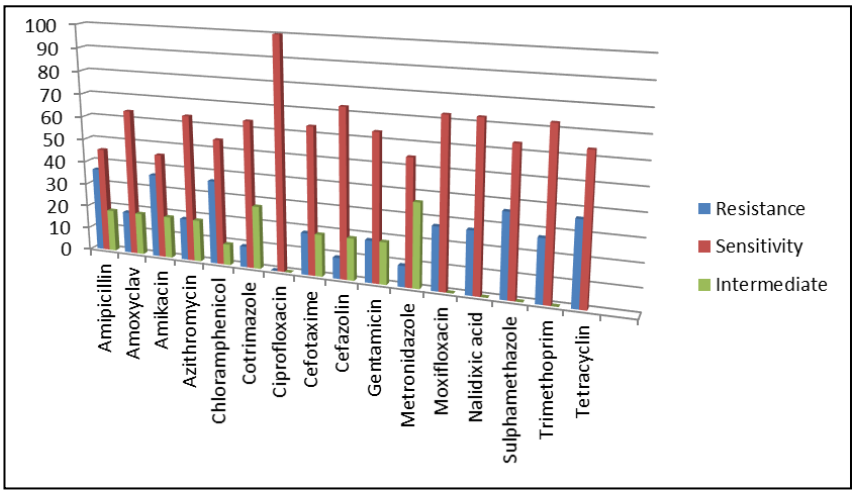

Fig 2: Percentage wise antibiogram of *E. coli* O157:H7 isolates from beef samples

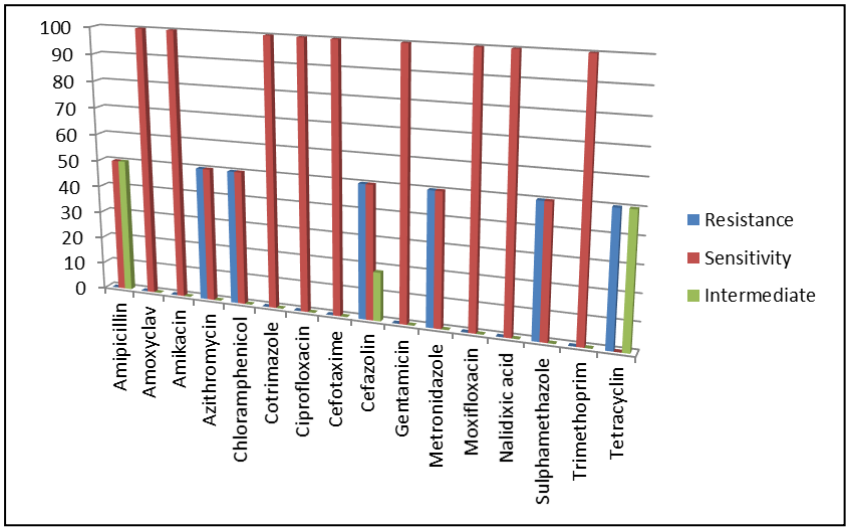

Fig 3: Percentage wise antibiogram of *E. coli* O157:H7 isolates from chevon samples

In the present study 42.8 per cent of isolates showed resistance to sulphamethazole and tetracycline, followed by 35.71 per cent of isolates showing resistance to chloramphenicol. It was also observed 28.5 per cent of isolates were resistant to amipicillin, amikacin and azithromycin, 21.43 per cent resistant to metronidazole, moxifloxacin, nalidixic acid and trimethoprim, 14.29 per cent resistant to amoxyclav, cotrimazole, cefataxim, cefazolin and gentamicin. None of the isolates showed resistance to ciprofloxacin. Khan *et al.* [16] reported the antibiogram of

EHEC isolates from different samples. They found 40 isolates from cattle and 4 from food samples in Kolkata were resistant to ampicillin and gentamicin. Durso *et al*. [7] observed that 17 (21.0 per cent) of the 81 *E. coli* O157:H7 strains and 11 (27.5 per cent) of the 40 *E. coli* strains from the raw beef meat samples were resistant to antibiotics like ampicillin, gentamicin, sulfamethoxazole and tetracycline. Johnson *et al*. [15] revealed that *E. coli* isolates from human diarrhea and poultry meat samples were resistant to trimethoprim sulfamethoxazole, nalidixic acid, cefataxim and cefazolin. Compared to the results of present study higher resistance was noticed by Momtaz and Jamshidi [20]. They observed that isolates of *E. coli* O157:H7 from the chicken meat products were resistant to tetracycline (76.82 per cent), followed by chloramphenicol (73.17 per cent). Croxen *et al*. [4] observed that Isolates of *E. coli* O157:H7 from human and bovine source were resistant to tetracycline. Furthermore, they observed, there was significant resistance to ampicillin and trimethoprim, which was more frequent in non-O157 EHEC isolates. Schmidt *et al*. [26] assessed the antimicrobial susceptibilities of all 150 third generation cephalosporin-resistant *E. coli* isolates from beef cattle production and processing continuum. They found the isolates were resistant to ampicillin, cefataxim, ceftriaxone, trimethoprim-sulfamethoxazole and nalidixic acid which were in agreement with the results of present study.

Although the antibiotic resistance profiling of the isolates were not worrying, the chances of bacteria acquiring resistance to more drugs cannot be ruled out as the transfer of antibiotic resistance from other bacteria to *E. coli* O157:H7 has been already established [26].

Biofilm production assay

A total of 14 PCR confirmed isolates of *E.coli* O157:H7 from the beef (12) and chevon (2) were screened for the biofilm production using the modified congo red agar. In that only 8 (57.14 per cent) isolates (7 from beef and 1 from chevon samples) were found to have the ability of biofilm production. The details about the biofilm producing *E. coli* O157:H7 isolates are shown in the table 9. The positive biofilm producing *E. coli* O157:H7 isolate is shown in the plate no. 4.

Table 9: Details of biofilm producing *E. coli* O157:H7 isolates

samples	Beef												chevon	
Isolates number	1	2	3	10	12	16	19	20	28	30	31	32	Ch15	Ch23
Ability of biofilm production	P	P	N	N	P	N	P	P	N	P	N	P	P	N

P: Positive**, N:** Negative

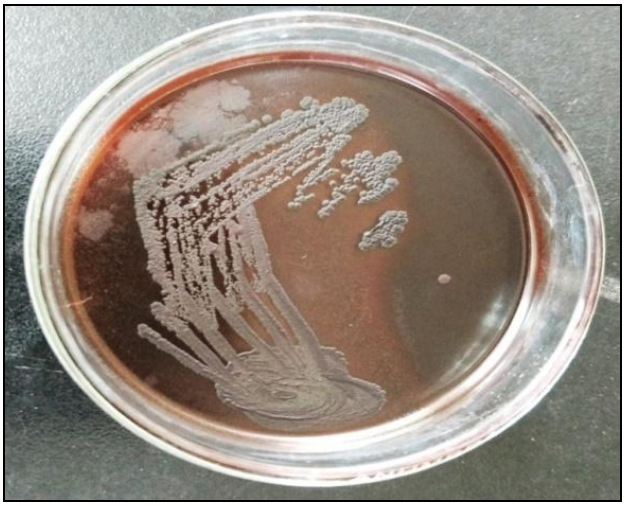

Plate 4: Biofilm producing *E.* Coli O157:H7 in Congo Red agar

In this study, a total of 14 PCR confirmed isolates of *E.coli* O157:H7 from the beef (12) and chevon (2) were screened for the biofilm production using the modified congo red agar. In that 8 isolates, 7 were from beef and 1 was from chevon sample. This result was comparatively higher than the study conducted by Fakruddin *et al*. [8] who reported that about 30.79 per cent of *E. coli* isolates as biofilm producers. The presence of biofilm producing EHEC O157:H7 in the present study (57.14 per cent) is comparatively lower than Dadawala *et al*. [5] who reported 85.71 per cent *E. coli* isolated as biofilm producers in congo red agar.

The results reveal that about 57.14 per cent of the isolates have the ability to produce biofilm. This is the matter of concern as this organism can resist normal cleaning practices in the slaughter house and in the kitchen. This may result in cross contamination of other meat during slaughter and other food in the kitchen including vegetarian food. This result clearly indicates the need for proper handling and processing of meat. It is also important that during slaughter and in kitchen whereever meat is prepared, care shall be taken to avoid cross contamination. Further studies are required to understand the behavior including resistance to cleaning by *E. coli* O157:H7 in biofilm.

Conclusion

This study findings suggests that cross-contamination of carcasses may be occurring in retail meat shops and due to higher prevalence in animals. Cattle act as a reservoir host for EHEC O157:H7 resulting in higher food contamination. Mainly in the beef retail shops beef carcasses were not hoisted and kept on the floor for dressing. During handling of the carcass the intestinal contents were spilled out. This may result in cross contamination of other meat during slaughter and other food in the kitchen including vegetarian food. This clearly indicates the need for proper handling and processing of meat. It is also important that during slaughter and in the kitchen where ever meat is prepared, care shall be taken to avoid cross contamination.

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