INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

Volume: 2: Issue-3: July-Sept -2011 IJABPT *ISSN 0976-4550*

PCR DETECTION OF '*STX* 1' AND '*STX 2*' TOXIGENIC GENES IN MULTIPLE ANTIBIOTIC RESISTANT *ESCHERICHIA COLI* POPULATION AND PHENOTYPIC DETECTION OF ESBL PRODUCING *ESCHERICHIA COLI.* ISOLATES FROM LOCAL VARIETY OF POULTRY.

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ABSTRACT: PCR detection of 'stx 1' and 'stx 2' toxigenic genes in multiple antibiotic resistant Escherichia coli population and Phenotypic detection of ESBL producing Escherichia coli isolated from traditional local variety of poultry was conducted The study revealed that out of 30 samples collected 20 were positive for E.coli All the 20 isolates were subjected for the detection of 2 toxin genes (stx1 & stx2 genes) by PCR. Only 3 isolates (L3, L4 & L5) gave rise to PCR product against its specific primers of stx1. All the isolates were negative for the presence of stx2 gene. Thus 15% of E.coli population under this study is potential isolates and indicates that healthy retail poultry birds are the carrier of E.coli pathogenic strains. The poultry birds may therefore, be considered as a major reservoir of E.coli.

Key words: PCR, E-coli, stx1, stx 2, toxin genes, poultry birds.

INTRODUCTION

Escherichia coli named after Theodor Escherich is a gram negative rod shaped bacterium that is commonly found in the lower intestine of warm blooded organism (endotherms). Most *E.coli* strains are harmless, but some such as serotype O157:H7 can cause serious food poisoning in humans and are occasionally responsible for product recalls (Barkocy-Gallagher et al.,2003). The harmless strains are part of the normal flora of the gut, and can benefit their host by producing vitamin K2 and by preventing the establishment of pathogenic bacteria within the intestine. *E.coli* is an ideal indicator organism to test environmental samples of fecal contamination (Bell et al., 1998; Primentel et al., 2000). *E.coli* is also present in the gut of every chicken as in the gut of mammals including humans. Even though bacteria form part of normal intestinal flora, the peaceful living together of chicken and *E.coli* can be disturbed in a disease called collibacillosis (Brittingham et al., 1988). The frequency with which antibiotic – resistant bacteria are encountered in our environment continues to be a cause of much concern. Although most of these resistant bacteria are themselves nonpathogenic, they pose the threat of potential transfer of their resistance factors to pathogenic species (Faddoul et. al., 1966). Furthermore, since antimicrobial drugs are used in plant and animal medicine, the spread of selected resistant organisms from one host to another may be important. The genetic information for antibiotic resistance is carried on R plasmids (R factors), which are pieces of extrachromosomal DNA (Johnson et al., 2002).

Multiple antibiotic resistant strains can be transferred from animal to human through contaminated food (Carter et al.,1990) reported that multiple resistant bacterial strains were transmitted to humans by raw meat and milk . Poultry feces are a potential source of antibiotic resistant bacteria. If released into the environment, resistant strains may contaminate water and food sources and can be a potential threat to human health (Chiu et al., 2004). *E.coli* are resistant to β-lactam antibiotics because of production of an enzyme called β -lactamase. The antibiotics are used to fight serious infection that *E.coli* bacteria can cause. The resistance can therefore be transferred when someone ingesting β-lactamases producing bacteria through contaminated water and food. Various types of β-lactamases have been distinguished which can inactivate different β-lactam antibiotics.The most dangerous are what are known as extended spectrum β – lactamases (ESBL) because they are the most efficient in breaking down a wide range of β -lactam antibiotics including the third generation antibiotics. Apart from antibiotic resistance factors, *E.coli* , particularly the pathogenic forms, also carry different toxigenic factors determined at genetic level. Some of the toxin genes identified in *E.coli* are *stx1, stx2, elt, est, hly, eae* etc. PCR is a powerful molecular biology technique that was introduces to facilitate the detection of virulence genes. Based on recently published prevalence data of virulence – associated factors in Avian pathogenic *E.coli* (APEC) and their rules in pathogenesis, PCR is used as a molecular tool supplementing current diagnostic schemes that mainly rely on serological examination of strains isolated from chicken droppings (Frank et al.,1998) . Considering the above the present study was undertaken with the objectives: 1] To isolate and identify *E.coli* in local chicken droppings collected from different local market places of Dhing, Nagaon (Assam) 2] To study the antibiogram of the isolates. 3] To detect (phenotypic) ESBL producing isolates.4] To detect prevalence of two toxin genes '*stx1*' and '*stx2*' in the *E.coli* isolates.

MATERIALS AND METHODS

Fresh chicken droppings were collected in sterile test tubes from local chickens at different traditional local market areas of North western part of Nagaon (Dhing , Moirabari, Dumdumia , Bechamari, Lahkar Bazar, Dhupguri Bazar, Mathkhula Bazar) in Nagaon district of Assam. Collected samples were then bring to the laboratory and immediately processed i. All the culture media used for the isolation of *E.coli* were sterilized at 20 lb pressure for 15 minutes. The identification of toxigenic genes from E.Coli isolates from local variety of poultry was done by PCR based technique. **Presumptive test for detection of coli forms:** Tubes containing 5ml of MacConkey broth with inverted Durham's tubes were inoculated with a loopful of fecal sample and incubated at 44.5°C .After 24 hr., the tubes were checked for acid and gas production. From the acid and gas positive tubes, a loopful of the culture was streaked directly onto MacConkey Agar plates. Small, pink colonies with circular form, convex elevation and entire margin were selected and processed for further analysis (Cruickshank et al., 1975 and Edwards and Ewing, 1986). *E.coli* cultures were maintained on nutrient agar slant and stored at refrigerated condition for the study period. Purity of the isolates was checked whenever felt necessary.

Morphological characteristics: *E.coli* cutures were subjected for Gram's staining test . A thin smear of cultures were prepared on a glass slide which was heat fixed and air dried. The smear was treated with crystal violet (1min.) then washed with water, Gram's iodine was then applied over the smear and allowed to stand for 1min. The slide was washed with water followed by gentle treatment with alcohol (10-15sec.) till the violet colour came off the slide. It was then immediately washed with water and counterstained with safranin (30sec). Finally the slide was washed with water, blotted dry and examined under oil immersion lens of a microscope. Motility test was done by hanging drop method. A saline suspension of the culture was prepared and a very small drop of the culture was placed on the centre of the cover slip. The cover slip adhered to the glass slide and the hanging drop was suspended in the well. The motility of the organism was observed under microscope.

Biochemical test: The cultures were subjected to IMViC test for biochemical characteristics. In Indole test, one loopful of fresh, overnight culture was inoculated into 3ml of 2% peptone medium and incubated at 37°C for 48hr. *E.coli* have the ability to hydrolyze tryptophan into indole, pyruvic acid and ammonia. After incubation, 0.5 ml Kovac's reagent was added to it and gently shaken. Cherry Red colour in the alcohol layer indicates positive test and this colour is produced by the reagent. Then the cultures were subjected for Methyl red test. Here, one loopful of fresh, overnight culture was inoculated into 3ml of glucose phosphate medium and incubated at 37°C for 48hr. After incubation, 5 drops of methyl red solution was added. At a pH 4, *E.coli* oxidized glucose to organic acid and the indicator turns red which shows a positive reaction. In Voges-Proskauer test, , One loopful of fresh, overnight culture was inoculated into 3ml of glucose phosphate medium and incubated at 37°C for 48hr. After incubation, 1ml of 40% KOH solution and 3ml of α-naphthol was added gently shaken and observed for up to 2hr. for the appearance of a pink colour which indicates a positive test. *E.coli* gives negative response to the medium. This indicates glucose is hydrolysed to organic acid but *E.coli* has no ability to oxidize organic acid to acetylmethyl carbinol and diacetyl compound. Then the cultures were subjected to Citrate utilization test. One loopful of fresh, overnight culture was inoculated in 3ml of Koser's citrate medium, incubated at 37°C and the incubation period continued upto 4 days. *E.coli* does not use citrate as a carbon source. So, it does not show any change in turbidity of the medium.

Confirmatory test: First, the cultures were subjected to E.M.B. Agar (Levine). E.M.B. Agar plates were prepared and streaked with isolated *E.coli* strains in Nutrient Agar slant and were incubated at 37°C for 24 hr. to obtain the result. Dyes Eosin Y and Methylene Blue reacts with products released by *E.coli* using lactose or sucrose as carbon and energy source, forming metallic green sheen. Then the cultures were subjected to MUG test which is 95% confirmatory test. A chromogenic or flurogenic glucoronide is incorporated into a conventional medium and enzyme activity detected by the production of colour or fluorescence. MUG plates were prepared and incubated for 2-3 hr. Thereafter, point inoculation of the isolated *E.coli* strains in Nutrient Agar slant was done and were then incubated at 37°C for 12-18 hr. after which they were observed under UV chamber.

Antimicrobial susceptibility testing (AST): AST was performed as per manufacturer's guidelines. A total of 9 antimicrobial agents (**Table-5**) were used in this study (Tortora, 2007)

Disc Diffusion Method (Kirby Bauer Method): Mueller Hinton Agar was prepared and poured into 90mm diameter sterile petridishes to a depth of 4 mm. A well isolated colony from an agar plate culture was selected .The top of the colony was touched with a loop and transferred to tube containing 4-5ml of 1% peptone broth. The culture was then incubated at 35°C until it exceeded the turbidity of 0.5 Mc Farland standards (2-6hr.). A sterile nontoxic cotton swab on a wooden applicator was dipped into a standardized inoculums and rotated firmly against the upper inside wall of the tube to express the excess fluid and streaked the plates for 3times, turning the plates at 60° angle. The inoculums were then allowed to dry for 5-15 minutes with lid in place. Using sterile forceps, appropriate antimicrobial discs were placed on the inoculated plates and disc to disc distance was maintained at 24mm. Within 15 mins of applying the discs plates were inverted and incubated at 35°C. After 16-18 hr. of incubation plates were examined using a zone reading ruler on underside of the plate, the diameter of each zone of inhibition in mm was measured and using manufacturer's interpretative chart (Himedia), the zone sizes of each antimicrobial disc was interpreted reporting the isolate as Resistant(R),or Sensitive(S).

Phenotypic Detection of ESBL production**:** It was done by AST test using oxy-immino antibiotic belonging to 3rd generation Cephalosporin viz. Cephotaxime, Ceftazidime and Cefpodoxime for primary screening of *E.coli.* Confirmatory test of ESBL production was done by Disc Diffusion Confirmatory test (DDCT).

Here, an antibiotic disc was placed at a distance of 3omm apart from another corresponding antibiotic supplemented with β-lactam inhibitor i.e. Ceftazidime and Ceftazidime/ Clavulanic acid, Cephotaxime and Cephotaxime/ Clavulanic acid. If the zone of inhibition produced more than 5mm when the antibiotic supplemented with β- laclam inhibitor was placed to that antibiotic placed alone, then that was considered positive - production of ESBL. The result was interpreted as per the guidelines of Clinical Laboratory Standard Institute (CLSI).

Genotypic detection of stx1 and stx2 genes by PCR

I) Template DNA preparation: A single colony was inoculated into Brain Heart Infusion broth (BHI, Himedia, INDIA).It was then incubated overnight at 37°C. 1.5 ml of broth culture was transferred into an eppendorf tube and centrifuged at 8000 rpm for 2mins. The supernatant was discarded and pellet was resuspended in 100 µl of autoclaved distilled water. It was then boiled for 10-15 minutes and immediately transferred to ice. The supernatant was used as DNA template.

II) PCR Amplification of virulent genes (*stx1 and 2*) and Analysis of the PCR products**:** The detection of these virulent genes were carried out as per the method described by Rahman (2002). PCR products were visualized in a horizontal submarine agarose gel electrophoresis as described by Sambrook et al. (1989).

OBSERVATION & RESULTS

A total of 30 dropping samples were collected from local chicken of different retail outlets of different local traditional market areas of Dhing in Nagaon (Assam) during a six month period. Out of 30 samples collected, 20 were lactose fermenting and produced acid and gas. Thus 20 samples were presumptive positive. Incidence of the total samples collected is found to be 66.67% (Table-2). Presumptive positive samples were further processed for isolation and confirmation of *E.coli* by morphological, biochemical and cultural procedures. All the 20 isolates were short, Gram negative and motile bacilli. All the isolates were indole and methyl red positive and VP and citrate negative, thereby giving ++ - - result for IMViC (Table-3). Hence biochemical characteristics suggested that they were *E.coli* isolates. All the isolates produced colonies with green metallic sheen on EMB plates and showed fluorescence in MUG test (Table-4 & Figure-1). Thus it was confirmed that all the 20 isolates were *E.coli.* All the isolates were susceptible to Imipenem (100%). High degree of sensitivity was observed in Getamycin (85%) and Ciprofloxacin (70%). All the 20 isolates were found to be resistant to all the 9 agents tested. The isolates were highly resistant to Amikacin (85%), Cefpodoxime(75%) followed by Ampicillin (70%)[Table-5]. This shows that 3(15%) isolates were resistance to 1 or 2 antimicrobial agents and 17 ((85%) isolates were resistance to 3 or more than 3 antimicrobial agents (MAR)[Figure-.2] Screening test for ESBL using two standard 3 oxy-immino antibiotics (Ceftazidime and Cephotaxime) indicates presence of 14 isolates resistant against Ceftazidime (30mcg) and 12 isolates resistant against Cephotaxime(30mcg)[Table-7]. The result of disc diffusion confirmatory test (DDCT) for ESBL indicate that only 1 isolate(5%) , L-1 is a confirmed ESBL producer, the clear zone increase in diameter by 9mm in presence of β-lactam inhibitor i.e. clavulanic acid(Figure-1).

Table 1: Antimicrobial agents Group and concentration per disc of the antimicrobial/antibiotic agent (Tortora, 2007)

S.No	Antibiotic Group	Antibiotic agent	Concentration(ug)
1	Penicillin(β-lactam)	Ampicillin,Imipenem	A-10,I-10
2	Cephalosporins	Cephotaxime, Cefepime,Cefpodoxime,Ceftazidime	Ce-30,Cpm-30,Cep-10,Ca-30
3	Aminoglycosides	Gentamycin,Amikacin	G-10,Ak-30
4	Quinolones	Ciprofloxacin	Cf-5

Table2. Results of Presumptive test and percentage of incidence of coliform positive samples.

S.No.	Sample identity	Sample accession no.	Presumptive test	Positive samples (%)
1	DH-1	L-1	+	
2	DH-2	L-2	+	
3	DH-3	L-3	+	
4	DH-4	-	-	
5	DH-5	L-4	+	
6	DHUP-1	L-5	+	
7	DHUP-2	-	-	
8	DHUP-3	L-6	+	
9	DHUP-4	L-7	+	
10	DHUP-5	-	-	
11	MOI-1	-	-	
12	MOI-2	L-8	+	66.67%
13	MOI-3	L-9	+	
14	MOI-4	-	-	
15	MOI-5	-	-	
16	BECH-1	L-10	+	
17	BECH-2	-	-	
18	BECH-3	L-11	+	
19	BECH-4	-	-	
20	BECH-5	L-12	+	
21	LAH-1	L-13	+	
22	LAH-2	L-14	+	
23	LAH-3	-	-	
24	LAH-4	L-15	+	
25	LAH-5	L-16	+	
26	DUM-1	L-17	+	
27	DUM-2	L-18	+	
28	DUM-3	-	-	
29	DUM-4	L-19	+	
30	DUM-5	L-20	+	

Table 3. Morphological characteristics and biochemical characteristics of *E.coli*

Serial No.	Sample accession no.	Gram's staining	Motility	Indole	Methyl Red	Voges Proskauer	Citrate
1	L-1	-	+	+	+	-	-
2	L-2	-	+	+	+	-	-
3	L-3	-	+	+	+	-	-
4	L-4	-	+	+	+	-	-
5	L-5	-	+	+	+	-	-
6	L-6	-	+	+	+	-	-
7	L-7	-	+	+	+	-	-
8	L-8	-	+	+	+	-	-
9	L-9	-	+	+	+	-	-
10	L-10	-	+	+	+	-	-
11	L-11	-	+	+	+	-	-
12	L-12	-	+	+	+	-	-
13	L-13	-	+	+	+	-	-
14	L-14	-	+	+	+	-	-
15	L-15	-	+	+	+	-	-
16	L-16	-	+	+	+	-	-
17	L-17	-	+	+	+	-	-
18	L-18	-	+	+	+	-	-
19	L-19	-	+	+	+	-	-
20	L-20	-	+	+	+	-	-

Table 4. Confirmatory Test for *E.coli*

S.No.	Sample accession no.	Green metallic sheen on EMB plates	MUG test (fluorescence)
1	L-1	+	+
2	L-2	+	+
3	L-3	+	+
4	L-4	+	+
5	L-5	+	+
6	L-6	+	+
7	L-7	+	+
8	L-8	+	+
9	L-9	+	+
10	L-10	+	+
11	L-11	+	+
12	L-12	+	+
13	L-13	+	+
14	L-14	+	+
15	L-15	+	+
16	L-16	+	+
17	L-17	+	+
18	L-18	+	+
19	L-19	+	+
20	L-20	+	+

Table 5. Antimicrobial Sensitivity of *E.coli*

S.No	Antimicrobial agents(ug/disc)	Resistant isolates (%)	Sensitive isolates (%)
1	Ampicillin(10)	14(70%)	6(30%)
2	Amikacin(30)	17(85%)	3(15%)
3	Cefpodoxime(10)	15(75%)	5(25%)
4	Ceftazidime(30)	14(70%)	6(30%)
5	Cephotaxime(30)	12(60%)	8(40%)
6	Gentamycin(10)	3(15%)	17(85%)
7	Cefepime(30)	11(55%)	9(45%)
8	Ciprofloxacin(5)	6(30%)	14(70%)
9	Imipenem(10)	0	20(100%)

Table6. Multiple Antimicrobial Resistant pattern of *E.coli*

S.No	Resistance to no. of antimicrobial agents	Sample accession no.	Number of isolate
1	No resistance	-	-
2	1	-	-
3	2	L-2,L-5,L-10	3(15%)
4	3	L-1,L-3,L-6,L-11	4(20%)
5	4	L-4	1(5%)
6	5	L-7,L-12,L-17,L-18,L-20	5(25%)
7	6	L-13,L-14,L-15,L-16	4(20%)
8	7	L-8,L-9,L-19	3(15%)

Table7.Screening test and Phenotypic Confirmatory test for ESBL production in E.coli isolates

Strain	Ca30 (zone of inhibition in mm)	R/S	Ce30 (zone of inhibition in mm)	R/S	Cac	Cac-Ca	Cec	Cec-Ce
L-1	28	S	4	R	-	-	13	9
L-2	26	S	31	S	-	-	-	-
L-3	26	S	30	S	-	-	-	-
L-4	21	S	26	S	-	-	-	-
L-5	32	S	36	S	-	-	-	-
L-6	25	S	29	S	-	-	-	-
L-7	8	R	28	S	12	4	-	-
L-8	14	R	10	R	17	3	12	2
L-9	12	R	10	R	13	1	13	3
L-10	13	R	25	S	14	1	-	-
L-11	10	R	27	S	12	2	-	-
L-12	11	R	10	R	12	1	11	1
L-13	12	R	8	R	14	2	10	2
L-14	13	R	5	R	17	4	8	3
L-15	14	R	8	R	17	3	9	1
L-16	12	R	10	R	13	1	12	2
L-17	10	R	12	R	13	3	13	1
L-18	6	R	8	R	10	4	11	3
L-19	5	R	4	R	8	3	5	1
L-20	7	R	2	R	8	1	4	2

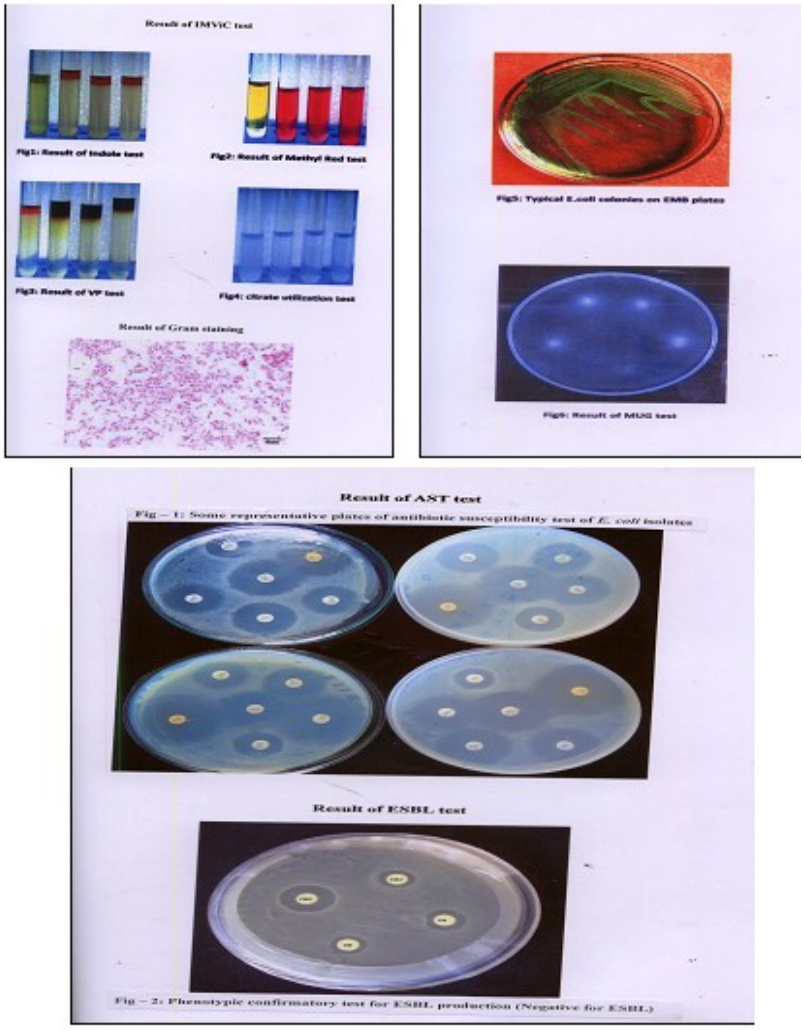

Figure :1- Photoplate of IMVIC,EMB, MUG, AST and esbl test

All the 20 isolates were subjected for the detection of 2 toxin genes (stx1 & stx2 genes) by PCR. Only 3 isolates (L3, L4 & L5) gave rise to PCR product against its specific primers of *stx1* **(**Figure-2). All the isolates were negative for the presence of *stx2* gene. Thus 15% of *E.coli* population under this study is potential isolates and indicates that healthy retail poultry birds are the carrier of *E.coli* pathogenic strains.

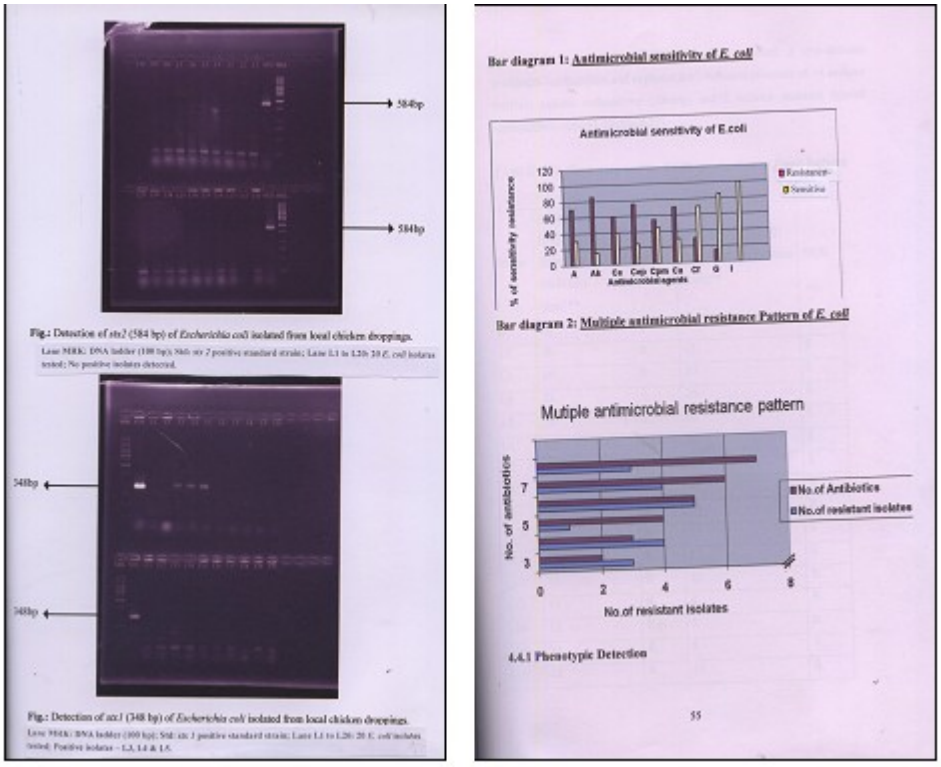

FIGURE: 2 –photo plate of Antimicrobial sensitivity test and PCR detection of stx1 & stx2 toxin genes.

SUMMARY AND CONCLUSION

Escerichia coli is an ubiquitous microorganism recorded in a wide variety of habitats. This microscopic bacillus is a store house of potent information. As they are ubiquitous in nature, it is recognized by all microbiologists particularly associated with surveillance programmes. *E. coli* is a common inhabitant of intestinal tract of poultry and is therefore can be a common source of *E. coli* for contamination of water, soil and food. Interestingly, high incidence of antibiotic resistance *E. coli* in poultry becomes an important source of transferring colonic bacteria to human being. This study shows high percentages of isolates are multiple antibiotic resistant and some of these may carry ESBL producing *E. coli* strains. ESBL producing *E. coli* strains also exist in the gut flora of poultry but with a very low incidence (5%). Some isolates carry toxigenic genes like *stx1* and *stx2* and therefore they can cause disease in poultry and human being. Poultry products are considered as good source of protein and considering the quantam of poultry products consumed each year one can easily imagine the importance of poultry products for people throughout the world. Unfortunately, due to high incidence of *E. coli* in the enteric system of poultry birds, they may be considered as a major reservoir of *E. coli*. Much of the poultry products may become a source of antibiotic resistance and toxigenic genes. ESBL producing *E. coli* strains existing in the gut flora of poultry is really very difficult to manage disease due to their infection. So, these make the product hazardous from the nutritional point of view. However, our study with poultry droppings indicate that incidence of ESBL producing *E. coli* strains in gut of poultry is very low (5%).

So good management practices and judicious use of certain antibiotics as growth promoter in poultry feeds can significantly reduce such risk and one can produce safe poultry products by decreasing the load of resistant *E. coli* in the environment, foods and in human enteric system. This study revealed that Out of 30 samples collected 20 were positive for *E.coli*. Incidence of the total samples collected is found to be 66.67%.Thus it appeared that most of the poultry birds of study area carry *E.coli* in their enteric system. The poultry birds may therefore, be considered as a major reservoir of *E.coli*. Higher incidence of *E.coli* in guts of poultry was reported by other workers (Smet et al., 2010; Vandemaele et al., 1986).

REFERENCES

1. Barkocy-Gallaghar, G.A.T.M.Arthur, M.Rivera- Betancourt, X.Nou, S.D.Shackelford, T.L.Wheeler, and M.Koomarie.Seasonal prevalence of shiga toxin –producing *Escerichia coli* including O157:H7 and non-O157serotypes and Salmonella in commercial beef processing plants.J.Food Prot.**66**:1978-1986.2003.

2. Bell, C. and A.Kyriakides. *E.coli*: A practical approach to the organism and its control in foods Blackie Academic and Professional London, United Kingdom.1998.

3. Brittingham, M.C., S.A.Temple, and R.M. Duncan. Survey of the prevalence of selected bacteria in wild birds.*J.Wildl.Dis*.24:299-307.1988.

4. Carter,G.R.Staining procedures and Diagnostic procedures in veterinary bacteriology and mycology,5th e G.R.Carter, and J.R.Cole,eds.Academic Press,Inc.San Diego,CA.522- 3.1990.

5. Chiu, C.H., L.H.Su, and C. Chu. Salmonella enterica serotype choleraesuis: epidemiology, pathogenesis, clinical disease, and treatment.Clin.Microbiol.Rev.17: 311-322.2004.

6. Cruickshank, R.;DUgid, J.P.; Marmoin, B.P.and Swan R.H.A.Medical Microbiology.12th ed.vol.I 1975.

7. Edwards, P.R. and Ewing, W.H.Identification of Enterobacteriaceae.4th edn. Burgess Publishing Company, Minnesota, USA.1986.

8. Faddoul, G.P., G.W.Fellows, and J.Braid. A survey on the incidence of Salmonellae in wild birds.Avian Dis.**10**:89-94.1966.

9. Franck, S.M., B.T.Bosworth, and H.W.Moon. Multiplex PCR for enterotoxigenic, attaching and effacing and shiga toxin-producinq *Escerichia coli* strains from calves J.Clin.Microbiol.**36**:1795-1797.1998.

10. Johnson,T.J.,C.W.Giddings,S.M.Horne,P.S. Gibbs. Location of increased serum survival gene and selected virulence traits on a conjugative R plasmid in an avian Escherichia coli isolate. Avian Dis.46:342-352.2002.

11. Primental, D., L. Lach, R.Zuniga, and D.Morrison.Environmental and economic costs of nonindigeneous species in the United States.BioScience**50**:53-65.2000.

12. Rahman, H (2002) Multiplex PCR for detection of *Stx* gene of *Escherichia coli* .Indian Journal of Medical Research, June2002, 115:251-254.

13. Sambrook, J.; Fritisch, E.F. and Maniatis, T.Molecular cloning: A laboratory manual.Cold Spring Harbour, New York.1989.

14. Smeth Annimieke; Bacterial ESBL resistance in poultry transferable to human bacteria. Veterinary and Agrochemical Research Centre2010.