INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-3, Issue-3, July-Sept-2012 Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

Received: 14th May-2012 *Revised: 17th May-2012* *Accepted: 20th May-2012*

Research article

MOLECULAR CHARACTERIZATION OF SHIGA LIKE TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) ISOLATES FROM BROILER AND LOCAL VARIETY OF POULTRY

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ABSTRACT: *Escherichia coli* isolates from different samples of droppings collected from the different farms of broiler and local poultry were characterized to confirm the virulence. *E. coli* isolates recovered from different farms were examined for presence of genes encoding patho groups such as shiga like toxin producing *Escherichia coli* (STEC), (*stx1/stx2*) by Duplex PCR and then analysed the PCR Amplicons by AGE. The three and four isolates of *E. coli* recovered from local and boiler poultry were STEC because of presence of the *stx1* and *stx2* genes. Presence of *stx1* and *stx2* genes clearly indicated these as prime cause of pathogenecity. Further, demonstration of STEC in poultry becomes a public health concern, as poultry are potential reservoir of such agents, which may cause extra intestinal diseases like haemolytic uremic syndrome and thrombocytopenic purpurea by *stx1 &2* and these toxins kill vascular endothelial cells.

Key words: Poultry - PCR -AGE- STEC-ETEC- *E. coli* - *stx1/stx2*.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), also known as verotoxin–producing *E. coli* (VTEC) comprises a serologically diverse group of pathogens that cause disease in humans and animals (Jaeger et al., 2000). STEC infections have been described in a wide range of both domestic and wild animals, but the natural pathogenic role of bacteria has been demonstrated only in young calves (diarrhea or dysentery), weaning pigs (oedema disease), and dogs (cutaneous and renal vasculopathyin grey hounds)(Wieler et al.,1998). Studies have demonstrated that these animals including poultry harboring STEC are also associated with human illness (Johnsen et al., 1998).*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 which may be systemic or enteric (Brittingham et al., 1988).The common feature of STEC is the production of shiga toxins (Stx) that are considered to be the major virulence factors. The virulence of any organism is dependent on an array of virulence factors that play a vital role in the pathogenic process of the organism viz., attachment, invasion, toxicity etc. According to Nataro and Kaper (1998), expression of these factors is controlled by virulence genes present in the *E.coli* genome which is controlled by two pathogenic configuration-virulence related plasmid and chromosomal pathogenecity island (PI) (Gibbs et al., 2009).The two main groups consist of Stx1, which is nearly identical to the toxin of *Shigella dysenteriae* type1 and Stx2, which shares less than 60per cent amino acid sequence with Stx1. The genetic information for the production of Stx1 and Stx2 is located in the genome of lambdoid prophages integrated in the STEC chromosome (Melton et al., 1998). Stx1 shows only little sequence variations (Zhang et al., 2002) but several variants of Stx2 with altered antigenic or biological characteristics have been reported (Scheutz et al., 2001, Melton et al., 1998). 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) .This study was undertaken to examine *E. coli* isolated from different faecal samples of poultry with for the presence of genes encoding various virulence factors.

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MATERIALS AND METHODS

From organized poultry farms of Nagaon, Assam, where a total of 30 samples of local and 22 samples of broiler poultry were collected between August-September, 2011 and bring to the laboratory and immediately processed. The samples were then sterilized at 20 lb pressure for 15 minutes. The identification of toxigenic genes from *E.coli* isolates of local and broiler variety of poultry was done by PCR based technique.

Isolation of Escherichia coli

E.coli samples were processed for isolation and identification of bacteria as per the method described earlier (Cruickshank et al., 1975 and Edwards and Ewing, 1986).

E.coli cultures were subjected for Gram's staining test for Morphological characteristics and IMViC test for Biochemical characteristics. The cultures were subjected to E.M.B. Agar (Levine) for confirmatory test of *E.coli*. The lactose fermenting colonies grown on Mac Conkey agar plate by incubation at 37ºC for 16-18 h under shaking condition. *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.

Antimicrobial susceptibility testing (AST)

Drug susceptibility was determined by the disc diffusion method (Bauer et al., 1966) using the following commercial discs (Hi-Media, Mumbai, India). Ampicillin, Imipenem (A-10 mg,I-10 mg), Cephotaxime, Cefepime,Cefpodoxime,Ceftazidime (Ce-30 mg,Cpm-30 mg,Cep-10 mg,Ca-30 mg), Gentamycin,Amikacin (G-10 mg,Ak-30 mg) Ciprofloxacin(Cf-5 mg).

Detection of *E. coli* virulence genes *(stx1, stx2)* by PCR

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 for PCR for screening of virulence genes.

PCR for the detection of virulence genes was performed using a thermal cycler for 30 cycles in a total final volume of 25ul containing 12.5 µl Master mix (Fermentas). The reaction mixture contained 2.5µl of template DNA, 2.5ml of 10×PCR buffer with 15mM MgCl2 (Genei, Bangalore), 2.0ml of dNTP containing2.5 mM each of the four deoxynucleotidetriphosphates, 0.125µl of each primers of 'vir' genes (Hysel India pvt. Limited) and 1U of TaqDNA polymerase (Genei, Bangalore). The primers sequences of the targeted virulence genes and their PCR conditions are presented in the Table 1. The PCR amplified products were separated by horizontal submarine agarose gel electrophoresis (Sambrook et al.,1989) with 1.5per cent (w/v) agarose gels containing 0.05% µg/ml ethidium bromide in 1×TAE [0.04MTris acetate (Sigma, St Louis, USA), 0.001M EDTA,*p*H 8.0] buffer along with 100 bp ladder (Takara, Shuzo,Otsu, Japan) as a molecular weight marker. Then 5µl of PCR amplicons and 1µl of 6X loading dye (Fermantas) (0.03% bromophenol blue) was loaded into the wells of the gel. A standard DNA marker 100bp DNA ladder (0.1 µg/ml) (Fermentas) was mixed with 6X loading dye loaded in one of the wells. The gel was run at constant current at the rate of 1-5V/cm of Agarose gel i.e. at 70 V, 105mA current for 80 min.s till the bromophenol blue of the gel loading buffer migrated more than 4/5th of the length of the gel. After staining with ethidium bromide (Sigma, USA), the gels were viewed with the help of a gel documentation system /transilluminator (Vilber Lourmat) and photographed. The detection of these virulent genes were carried out as per the method described by Rahman (2002).

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Table 1: Polymerase chain reaction (PCR) primers and conditions used for the detection of *stx1 & stx2* gene in *E.coli* isolates

Toxin genes	Primer sequence	Amplicon size (bp)	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
stx1	F:5`-CAGTTAATGTCGTGGCGAAGG-3` R:5`-CACCAGACAATGTTAACCGCTG-3`	348bp	94°C(5min.s)	94°C(1min.)	55°C(1min.)	72°C(2min.s)	72°C(10min.s)
stx2	F:5`-ATCCTATTCCCGGGAGTTTAC-3` R:5` -GCGTCATCGTATACACAGGAG-3`	584bp	94°C(5min.s)	94°C(1min.)	55°C(1min.)	72°C(2min.s)	72°C(10min.s)

F-Forward primer
R-Reverse primer

OBSERVATION AND RESULTS:

This study revealed that out of 30 and 22 samples collected from local and broiler chicken of different farms of Nagaon (Assam) during a two months period. 20 were positive for *E.coli* i.e. lactose fermenting and produced acid and gas. Incidence of the total samples collected is found to be 66.67% & 90.91% respectively.
The bacteriological examination of broiler and local poultry droppings revealed the presence of Gram-negative bacilli. In the biochemical tests, the isolates were identified as *E coli.* The Antibiotic susceptibility pattern of the *E. coli* isolates showed narrow zone of susceptibility against different antibiotics, typical for the STEC. The antimicrobial susceptibility assay in the present study showed that most of the isolates were susceptible to Imipenem, Gentamycin, Ciprofloxacin, and resistant to Amikacin, ampicillin, Ceftazidime. Multidrug resistance was also previously reported from local and broiler droppings (Monoj et al., 2011).
The isolates were examined for presence of genes encoding various virulence factors associated with path groups such as STEC (*stx1/stx2*) by PCR. All the three and four isolates of local and broiler poultry harboured stx1and *stx2* genes respectively, as they gave348and 584bp amplicons, respectively (Fig. 1&2).

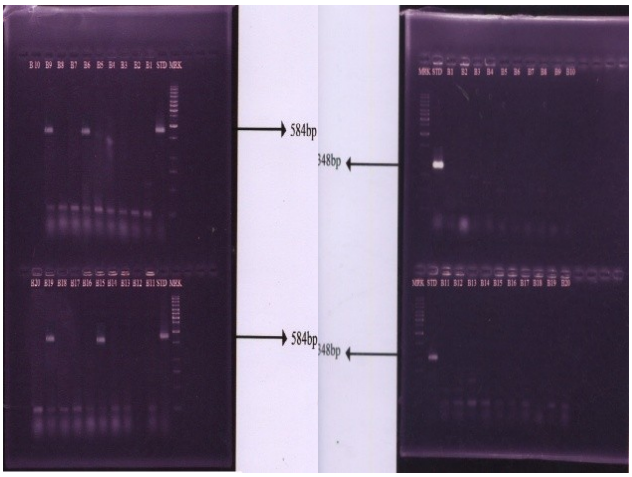

Fig.1: Detection of *stx2 &1*(584bp, 348 bp) of *Escherichia coli* isolated from broiler chicken droppings.
Lane MRK: DNA ladder (100bp); Std*: stx2 &stx1* positive Standard strain; Lane B1 to B20:20 *E.coli* isolates tested; Positive isolates detected for *stx2*: B6, B9, B15&B19 & No positive isolates detected for *stx1*.

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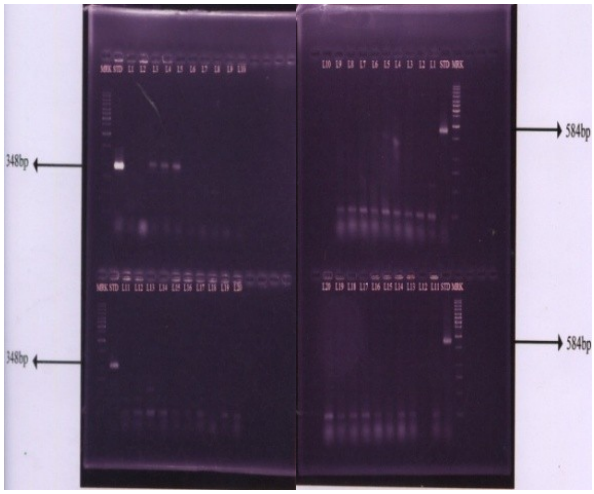

Fig.2: Detection of *stx1 & stx2* (348bp, 584bp) of *Escherichia coli* isolated from local chicken droppings.

Lane MRK: DNA ladder (100bp); Std: *stx1 &2* positive standard strain; Lane L1 to L20:20 *E.coli* isolates tested; Positive isolates detected for *stx1*: L3, L4&L5 & No Positive isolates detected for *stx2.*

All the 20 isolates were subjected for the detection of 2 toxin genes *(stx1 & stx2* genes) by PCR.
The organisms that give rise to PCR product of 348bp, 584bp were taken as positive for the presence of *stx1 &2* genes respectively. Only three isolates (L3, L4 & L5) and four isolates (B6, B9, B15&B19) gave rise to PCR product against its specific primers of *stx1 and stx2* **(**Figure-1&2). All the isolates of local variety of poultry were negative for the presence of *stx2* gene and broiler varieties of poultry were negative for stx1 genes. Thus 15% and 20% of *E.coli* population under this study is potential pathogenic isolates and indicates that healthy retail poultry birds are the carrier of *E.coli* pathogenic strains.

SUMMARY AND CONCLUSION:

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 through contaminated food i.e. raw meat and milk. Poultry feces are a potential source of antibiotic resistant bacteria.Toxigenic genes like *stx1* and *stx2* can cause disease in poultry and human being. This study revealed that Out of 30 and 22 samples collected 20 were positive for *E.coli*. .The characteristic attaching and effacing (A/E) lesion caused by most of the STEC depends on the activity of the multiple genes in the LEE including the type III secretion system and on the initiation of the signal transduction events (Delahay et al.,2001). Three STEC isolates (L3, L4 & L5) and Four STEC isolates (B6, B9, B15&B19) gave rise to PCR product against its specific primers of *stx1 and stx2* as determined in the Duplex-PCR. (Fig.1&2).Poultry may be important reservoir for these organisms. It is thus important to make an extensive survey on STEC in poultry. Molecular characterization has proved to be a rapid and accurate diagnostic approach for establishing the epidemiology of STEC. This study shows high percentages of broiler variety of poultry containing toxigenic gene. Incidence of the total samples collected is found to be 66.67% and 90.91% respectively. Thus it appeared that most of the poultry birds of study area carry *E.coli* in their enteric system. The major virulence factors of enterohaemorrhagic *E.coli* (EHEC) is the Shiga toxin (Stx) production that is absorbed into blood and causes systemic vascular damage resulting in edema disease(Moon et al.,2003) and hemorrhagic colitis.

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This toxin exists in two antigenic forms: Stx1 and Stx2 are encoded by *stx1* and *stx2* genes respectively (Sears and Kaper, 1996) which may cause extra intestinal diseases like hemolytic uremic syndrome and thrombocytopenic purpurea .So good management practices reduce the fecal contamination and one can produce safe poultry products .There is an urgent need to evaluate the poultry biodiversity of India and to compile a viable data base for future potential applications in scientific and biotechnological applications of food production. To achieve this objective, trained human resources in the areas of conservation biology especially genetics are to be made available within a reasonable time. The latest emerging genetic tools like DNA profiling procedures are real boons for such purposes and should be employed to the advantage of conservation genetics. Active involvement of various agencies and poultry community is very essential.

ACKNOWLEDGEMENT

I am grateful to principal Dhing College Nagaon, Assam for providing facilities to carry out the Project Work and also thankful to DBT for providing funds and infrastructure to carry on my work

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