RESEARCH ARTICLE

Molecular Detection of Virulence Genes among *Escherichia coli* Isolated from Poultry Faecal Samples

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) is known as a crucial zoonotic food-borne pathogen. The main virulence factor of STEC is the production of Shiga toxins 1 and 2. A total of 400 poultry faecal samples were collected from different poultry farms in and around Tirupathi, Andhra Pradesh. The samples were cultured in selective and differential culture media, and the virulence genes of *E. coli* isolates were analyzed by PCR assay. Out of 400 poultry faecal samples, 170 isolates were isolated and confirmed as *E. coli* by different biochemical tests with an overall prevalence of 42.5%. Out of 170 *E. coli* isolates 154 (90.5%) were found positive by targeting *uspA* gene with an amplification of 884 bp. All the 154 PCR confirmed *E. coli* isolates subjected to multiplex PCR for the detection of virulent genes targeting *eae A*, *stx 1* and *stx 2* and obtained an amplification products, respectively at 384 bp, 180 bp and 254 bp. Out of 154 *E. coli* isolates, 54 (35.06%) isolates had *eae A* gene, 36 (23.37 %) had *stx 1* gene, 41 (26.62 %) isolates had *stx 2* gene, 16 (3.89 %) isolates had both *eae A* & *stx 2*, and 12 (7.79%) isolates had both *stx 1* & *stx 2*. By avoiding contact with contaminated faeces and by maintaining proper hygienic measures at farm, contamination of poultry and poultry products may be avoided which in turn may safeguard the health of public.

Key words: *Escherichia coli*, Faecal samples, PCR, Shiga toxin, STEC.

Ind J Vet Sci and Biotech (2024): 10.48165/ijvsbt.20.5.25

Introduction

Escherichia coli is a facultative anaerobic, rod-shaped, Gram negative bacteria with many facets belonging to the family *Enterobacteriaceae*. According to the modified Kauffman scheme, *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigenic profile. A specific combination of O and H antigens defines the "serotype" of an isolate (Nataro and Kaper, 1998). Based on virulence factors, *E. coli* is classified as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), attaching and effacing *E. coli* (AEEC), and Shiga toxin producing *E. coli* (STEC) etc. (Bavaro, 2012).

Pathogenic strains of *E. coli* are more frequently linked to outbreaks of food borne disease. It is transmitted via faeco-oral route and commonly found in water, soil, and food (Waturangi *et al.*, 2019). It is now widely acknowledged that the *E. coli O157:H7* (STEC) strain is a significant contributor to food borne disease (Ramos *et al.,* 2020) and contaminated food and the faeces of asymptomatic reservoirs are the sources of the disease. Poultry meat is the food of the animal source most closely linked to human ExPEC infections (Nielsen *et al.,* 2020). The ExPEC group primarily associated with nosocomial and community-associated infections. Furthermore, it has been shown that the APEC and ExPEC strains, cause disease in poultry and humans, respectively, and can share genetic characteristics (Stomberg *et al.,* 2017). The information on the prevalence of *E. coli* from poultry faecal samples in Tirupathi, Andhra Pradesh, India is limited, hence the present study was aimed to isolate confirm and molecular characterize the *E. coli* isolates from the faecal samples of poultry by conventional methods and PCR including detection of virulence genes among the *E. coli*.

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How to cite this article: Padmavathi, T. V., Ramya, P., Swetha, C. S., Rani Prameela, D., Suresh, Y., & Tirupathi Reddy, E. (2024). Molecular Detection of Virulence Genes among Escherichia coli Isolated from Poultry Faecal Samples. Ind J Vet Sci and Biotech. 20(5), 130-133.

Source of support: Nil

Conflict of interest: None

Submitted 24/06/2024 **Accepted** 24/07/2024 **Published** 10/09/2024

Materials and Methods

The faecal samples were collected with sterile cotton swabs directly from the cloaca of chicken from different poultry farms in and around Tirupati. The samples were collected in sterile Ziplock bags and transported in an ice box within 2 h to the laboratory for further processing. The necessary

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precautions were taken while performing each procedure in a biosafety cabinet. Loop full of inoculums were transferred to Buffered peptone water tubes and were incubated at 37°C for 24 h. After incubation a loop full of inoculum was streaked on MacConkey agar and Eosin Methylene Blue agar plates and the plates were incubated at 37°C for 24 h. The individual colonies were identified by morphology and Gram's stain under Direct microscopy. The recovered isolates were further confirmed by biochemical tests like catalase test, mannitol motility test, nitrate reduction test, triple sugar iron agar test, urease test and IMViC tests (++--) as per the methods described in Bergey's manual of systemic bacteriology (1984). *Escherichia coli* isolates were further confirmed by using *E. coli* species specific-uniplex PCR targeting *uspA* gene. The DNA was extracted from culturally confirmed isolates by using Boiling and Snap chilling method.

PCR amplification was optimized in 25 µL PCR reaction mixture, consisting of PCR master mix (2X) (G-biosciences) 12.5 µL, forward & reverse primer (10 pmol/µL) 1.0 µL each, Template DNA 2.5 µL and Nuclease free water 8.0 µL. PCR assay was performed in micro-centrifuge thermal cycler with heated lid under standardized cycling conditions**.** The cycling conditions were standardized at 5 min initial denaturation at 94° C followed by 30 cycles of denaturation at 94° C for 1 min, annealing at 55° C for 1 min, initial extension at 72 ° C for 2 min and a 5 min final extension at 72°C followed by maintenance at 4°C. The primers used in the study were custom synthesized by Eurofins genomics India Private Limited, Bangalore. The resultant PCR products were subjected to 1.5% agarose gel electrophoresis as described by Sambrook and Russel (2001). The PCR product size was determined by comparing with a standard molecular weight marker. A simplex PCR and multiplex PCR assay were used for the detection of species specific gene *uspA* and virulence gene of *E. coli* (*eaeA, stx-1, stx-2*) respectively (Table 1 & 2). The DNA standard of *E. coli* (ATCC 25922) which carried all the three virulent genes under study was used for assessing the correct annealing temperatures. A 30 cycle PCR with annealing temperature of 59°C for 45 sec was found optimum for amplification of *eae A, stx 1* and *stx 2* genes with amplicons of 384 bp, 180 bp and 254 bp, respectively.

Results and Discussion

In the present study, out of 400 poultry faecal samples, 170 (42.5%) samples were positive on cultural isolation suggestive of *Escherichia coli,* showing pink colour colonies on MacConkey agar and metallic green sheen colonies on EMB agar**.** Almost similar prevalence of *E. coli* (43.6%) was reported by Byomi *et al*. (2017). In contrast to the present study, a higher prevalence of *E. coli* (55.6 to 100%) from poultry faecal samples was reported by Ramya *et al*. (2019) and Assoumy *et al*. (2021) and a lower one (22.4 to 38.5%) by Kalin *et al*. (2012) and Hassen *et al*. (2020) and a very low prevalence of 1.43% was reported by Wang *et al*. (2014). These differences in the prevalence rates of *E. coli* may be due to seasonal variations, poor biosecurity or hygienic conditions from area to area and from farm to farm. Further this may also attribute to variations in the strains causing pathogenicity and immunological status of the flocks (El seedy *et al.,* 2019)

All the culturally confirmed *E. coli* isolates were subjected to PCR by targeting *uspA* gene (Universal Stress Protein involved in actions such as adhesion and motility) for confirmation of the *Escherichia coli*. Out of 170 isolates targeted for *uspA* gene, 154 (90.5%) isolates were confirmed as *Escherichia coli* on molecular detection (Fig. 1). Ilcebaylik and Turkyilmaz (2020) reported 100% prevalence of *uspA* gene among the culturally positive *E. coli* isolates, whereas lower prevalence of species specific *uspA* gene was reported by Hardiati *et al*. (2021) and Fazal *et al*. (2022) as 55.6% and 78.0%, respectively. The *uspA* gene was found to be present in all strains of *E. coli* and therefore, recognized as a species-specific marker for all strains of *E. coli*.

Table 1: Details of the species-specific oligonucleotide primers used in this study

Target gene	Primer sequence 5′ – 3′	Amplicon size (bp)	Reference
uspA	F5′- CCGATACGCTGC-CAATCAGT- 3′ R5′- ACGCAGACCGTAAGGGC-CAGAT-3′	884bp	Chen and Griffiths (1999)

Table 2: Details of the primers used for detection of *stx 1*, *stx 2* and *eae A* genes

Target gene	Primer sequence 5′ – 3′	Amplicon size (bp)	Reference
eaeA	F5′- GACCCGGCACAAGCAT-AAGC- 3′ R5′- CCACCTGCAGCAACAA-GAGG-3′	384	Paton and Paton (1998)
stx1	F5′ATAAATCGCCATTCGTTGAC TAC-3′ R5′-AGAACGCCCACTGAGA-TCATC-3′	180	
stx2	F5′-GGCACTGTCTGAAACT-GCTCC-3′ R5′-TCGCCAGTTATCTGA-CATTCTG-3′	254	

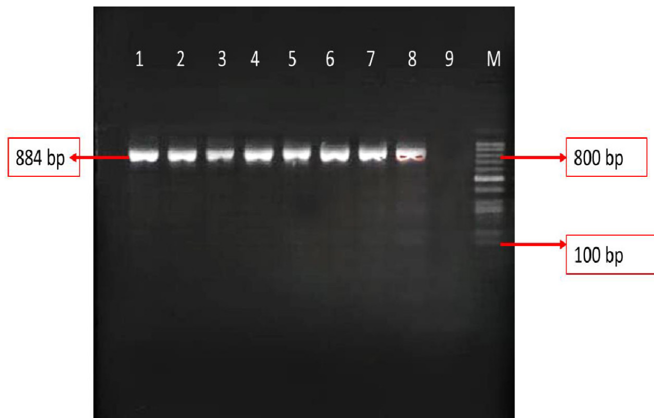

Fig. 1: PCR results of *Escherichia coli* isolates targeting *uspA* gene: Lane M: Molecular weight marker (100 bp), Lane 1-8: Amplified DNA of *E. coli uspA* gene (884 bp), Lane 9: Negative control (PCR reaction mixture without DNA template).

All the PCR confirmed *E. coli* isolates were subjected to multiplex PCR by targeting virulence genes *eae A, stx 1* and *stx 2* (Fig. 2). Out of 154 PCR confirmed *E. coli* isolates 54 (35.06%) were found to have *eae A* gene, 36 (23.37 %) were found to have *stx 1* gene, 41 (26.62 %) isolates had *stx 2* gene, 16 (10.38% %) isolates had both *eae A* & *stx 2*, and 12 (7.79%) isolates had both *stx 1* & *stx 2*. In contrary Ramya *et al.* (2019) reported *stx 1*, *stx 2* and both *stx 1 and stx 2* genes in 18.4, 52.6 and 28.9%, respectively, among the *E. coli* isolates of poultry faecal samples. Although, Chandran and Mazumder (2014) reported a lower prevalence of *stx 2* gene (23%) and absence of *stx 1*. The prevalence of *stx 1* in the present study was lower than the reports (42.9 and 68.1%) of El-Jakee *et al.* (2012) and Amir *et al.* (2021). In contrary, a very low prevalence of 1.1% was reported by Parvej *et al.* (2020).

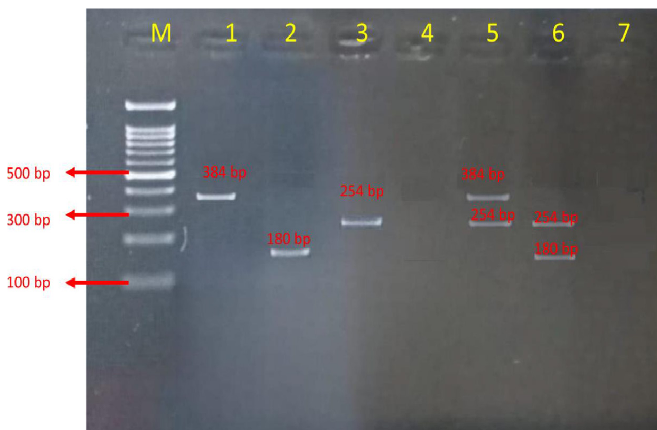

Fig. 2: Results of virulence genes of *E. coli* from poultry faecal samples by Multiplex PCR: Lane M: Molecular weight marker (100 bp), Lane 1: Amplified DNA of *eae A* gene (384 bp), Lane 2: Amplified DNA of *stx 1* gene (180 bp), Lane 3: Amplified DNA of *stx 2* gene (254 bp), Lane 5: Amplified DNA of *eae A* (384 bp) and *stx 2* (254 bp) genes, Lane 6: Amplified DNA of *stx 1* (180 bp) and *stx 2* (254 bp) genes.

Strains of *E. coli* carrying virulence genes like *eae A* (attaching and efficacing of enterocyte), *stx 1* and *stx 2* (Shiga toxin, surface localization of nucleolin and cytotoxic effect) are considered to be pathogenic (Pakbin *et al.*, 2021). Cattle, sheep and other animals are considered as the main reservoirs for *eae A*, *stx 1* and *stx 2* genes, whereas in poultry, Wani *et al.* (2004) reported a very low prevalence of *eaeA, stx 1*and *stx 2* genes.

Out of 154 PCR confirmed *E. coli* isolates, 54 (35.0%) harboured *eae A* gene. Almost similar prevalence (35.71%) was reported by El-Jakee *et al.* (2012). However the higher prevalence (46-68%) was reported earlier by Byomi *et al.* (2017) and Amir *et al.* (2021), whereas lower prevalence of 15-25% was reported by Dutta *et al.* (2011), Parvej *et al.* (2020) and Chandran and Mazumder (2014). Interestingly a very low prevalence of 2.74% was reported by Wani *et al.* (2004).

The present study reported a higher prevalence of *stx 2* (26.6%) than *stx 1* (23.37%). Similar types of results were reported by Ramya *et al.* (2019), whereas Kiranmayi and Krishnaiah (2011) reported higher prevalence of *stx 1* than *stx 2*. The variations in distribution of *stx* toxins among the animal species might be due to the genetic makeup of the species, feeding habits and behaviour according to the varied environmental conditions (Ateba and Mbewe, 2011). Griffin (1995) reported that *E. coli* with *stx 2* is more important than *stx 1* in the development of HUS and the strains that express *stx 2* alone are more likely to be associated with the progression to HUS than strains producing both *stx 1 and stx 2*.

The present study has recorded the lower prevalence or similar percentages of *E. coli* with virulence genes, which may be due to the reason that apparently healthy birds or birds with no history of diarrhoea were tested and this is in accordance with the reports of Dutta *et al.* 2011).

Conclusion

This study emphasizes the prevalence of virulent *Escherichia coli* in poultry faecal samples in and around Tirupathi, Andhra Pradesh, India. This may be responsible for cross contamination of chicken and its by-products, which may further leads to STEC infection in human. Therefore, careful processing of chicken and its products is an important public health measure, so that we may curtail the entry of these organisms into meat, thereby safeguarding the human beings from health hazards.

Acknowledgement

Authors are very much thankful to Dept. of Veterinary Microbiology and Dept. of VPHE, College of Veterinary Science, Tirupathi for providing facilities to conduct this research programme.

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