RESEARCH ARTICLE

Molecular Characterization of Avian Pathogenic *Escherichia coli* Isolates using Virulence Associated Genes

Bhargav B. Limbachiya, Rafiyuddin A. Mathakiya*, Esha Sinha, Anik M. Mathakiya

Abstract

Among various diseases, colibacillosis is one of the most important diseases, which causes heavy economic losses in broilers as well as in layers. It is caused by pathotype avian pathogenic *E. coli* (APEC). The present research work was carried out to detect and characterize the 60 *Escherichia coli* isolates. All the preserved isolates were obtained from various poultry farms located in and around Anand, Gujarat. All the isolates were revived and confirmed by polymerase chain reaction using *phoA* gene followed by characterization of the isolates based on seven virulence associated genes, *viz., astA, iss, vat, papC, iucD, irp2* and *tsh*. Out of the 60 *E. coli* isolates analysed, the overall highest positive percentage was for gene *iss* (93.34%), followed by *iucD* (60.00%), *irp2* (46.67%), *astA* (40%), *tsh* (33.33%), *vat* (26.67%) and *papC* (15.00%), respectively.

Keywords: Avian pathogenic *Escherichia coli* (APEC), Polymerase chain reaction (PCR), Virulence associated genes (VAGs).

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Introduction

Extra intestinal pathogenic *E. coli* (ExPEC) are relative pathogens originating from the normal intestinal flora, responsible for parenteral infections, which include various pathotypes like: Uropathogenic *E. coli* (UPEC), Neonatal meningitis *E. coli* (NMEC), Sepsis associated *E. coli* (SEPEC) and Avian pathogenic *E. coli* (APEC) (Sarowska *et al.,* 2022). APEC is the causative agent of avian colibacillosis and leads to huge economic losses in the poultry industry through morbidity, mortality and carcass condemnation (Mehat *et al*., 2021). Colibacillosis is characterized by septicemia, swollen head syndrome, yolk sac infection, cellulitis, as well as inflammation of different organs such as pericarditis, air sacculitis, and perihepatitis (Rezatofighi *et al*., 2021).

APEC harbours virulence-associated genes (VAGs), which encodes for adhesins (*fim*, *pap*), toxins (*ast*, *stx*), iron acquisition systems (*iuc, aer*), autotransporters, sugar metabolism, serum resistance proteins (*iss*), cell surface hydrophobicity, resistance to phagocytosis (*col*), production of cytotoxic necrotizing factor, K1 antigen, haemolysin production (*hly*), capsule and lipopolysaccharides complex (Mbanga and Nyararai, 2015). The virulence-associated genes are linked to pathogenicity islands and are found on large transmissible plasmids which can be transferred to non-pathogenic strains like avian fecal *E. coli* (AFEC) by the mechanism of horizontal gene transfer. Some virulence associated genes found in APEC, are also present in human pathogenic *E. coli*, and foodborne urinary tract infections (Markland *et al*., 2015). Keeping in mind the impact of APEC on poultry and human health, this research work was undertaken with the objective of molecular detection and characterization of avian pathogenic *E. coli* isolates based on virulence associated genes.

Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand-388001, India

Corresponding Author: Rafiyuddin A. Mathakiya, Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand-388001, India, e-mail: drramathakiya@gmail.com

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Materials and Methods

Revival of Avian Pathogenic *E. coli* Isolates

A total of 60 isolates of *E. coli* were used in the present study. These were isolated from different poultry farms located in and around Anand, Gujarat and were preserved at the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand, Gujarat. These isolates were initially stored in glycerol at -40°C. The isolates were plated onto MacConkey agar and were incubated at 37°C for 24 h. From each plate of MacConkey agar, the lactose fermenting colony was inoculated on Eosin methylene blue (EMB) agar and incubated overnight at 37°C for preliminary identification. The colonies on EMB agar showing greenish metallic sheen were presumptively considered *E. coli*.

The pure cultures of *E. coli* isolates were stored in Brain heart infusion agar (BHI) slants for further identification and characterization.

DNA Extraction of *E. coli* Isolates

The fresh suspension of *E. coli* was made in 100 µL of Milli-Q water by picking up a typical colony in a 200 µL PCR tube. The suspension was boiled at 95°C for 15 min and all cell debris were removed by centrifugation at 7000 x g for 1 min, and 3 µL of the supernatant was used as template DNA. The quality and quantity of DNA were checked on Nanodrop Spectrophotometer. An optical density (OD) 1 corresponds to 50 µg/mL of DNA. A ratio of 260:280 provides the proof of the purity of the nucleic acid. Pure preparations of DNA have a 260:280 ratio of 1.8.

Molecular Detection and Characterization of *E. coli* Isolates

All 60 *E. coli* isolates were confirmed using PCR for specific gene with optimum steps of thermal cycling condition as per Hu *et al.* (2011). For characterization of the isolates virulence associated genes were studied using different sets of primers, *viz.,* Enteroaggregative toxin (*astA*), Increased serum survival protein (*iss*), Iron repressible protein (*irp2*), P fimbriae (*papC*), Aerobactin (*iucD*), Temperature sensitive haemagglutinin (*tsh*), and Vacuolating transporter toxin (*vat*). The PCR amplification which targeted *astA*, *iss, irp2, papC, iucD, tsh,* and *vat* genes was carried out using primers as described by Ewers *et al.* (2004). The amplified PCR products were analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV light in a gel documentation system.

Results and Discussion

All 60 isolates produced pink colour colonies on MacConkey agar and showed greenish metallic sheen on EMB agar. All 60 *E. coli* isolates were confirmed by *phoA* gene-based PCR which yielded the desired amplicon size of 720 bp (Fig. 1). The result of present study agrees with the report of many investigators who confirmed 100% isolates as *E. coli* using *phoA* gene.

The PCR assay was performed for detection of individual virulence associated genes of APEC namely *astA*, *iss, vat, papC, iucD, irp2* and *tsh* using specific primer pair which yielded expected product size of 116 bp, 309 bp, 981 bp, 501 bp (Fig. 2), 714 bp, 413 bp and 824 bp, respectively. The prevalence of VAGs viz. *iss, iucD, irp2, astA*, *tsh, vat* and *papC* and were 56 (93.34%), 36 (60.00%), 28 (46.67%), 24 (40.00%), 20 (33.33%), 16 (26.67%) and 9 (15.00%), respectively among the 60 isolates (Table 1).

The *iss* gene encodes for virulence factor protectins. These protectins are typical for APEC and contribute to survival and proliferation of microorganisms in the host as its product plays an important role in inhibition of the complement system (Kocúreková *et al*., 2021). The result of present study for *iss* gene frequency (93.34%) is in the agreement with Oh *et al*. (2011), Arabi *et al*. (2013), Ammar *et al*. (2015), Elsayed *et al*. (2015) and Subedi *et al*. (2018), who detected *iss* gene from 90% to 100%. Ahmed *et al*. (2013) also detected *iss* gene @ 80.20%. On the contrary, Li *et al*. (2015), Paixao *et al*. (2016) and Roseliza *et al*. (2017) detected lower prevalence of 42.50%, 33.07%, and 51.20%, respectively.

Table 1: Virulence associated genes (VAGs) present in *E. coli* isolates

Name of Virulence Associated Genes	Prevalence of VAGs
iss	56/60 (93.34%)
iucD	36/60 (60.00%)
irp2	28/60 (46.67%)
astA	24/60 (40.00%)
tsh	20/60 (33.33%)
vat	16/60 (26.67%)
papC	9/60 (15.00%)

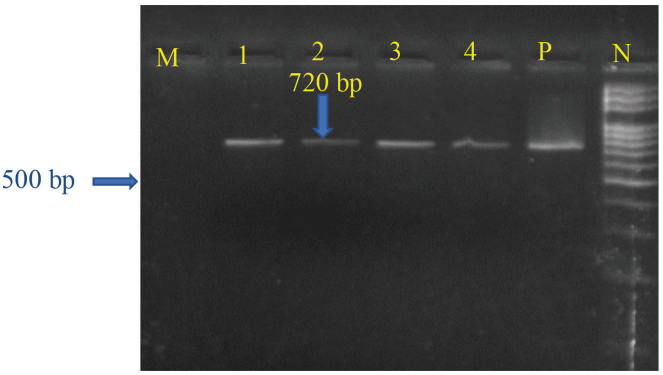

Fig. 1. Agarose gel showing amplified product for *phoA* gene of *E. coli* isolates (approx. 720bp)
M: DNA ladder – 1000 bp 1-4: Isolates (Samples)
P: Positive control N: Negative control

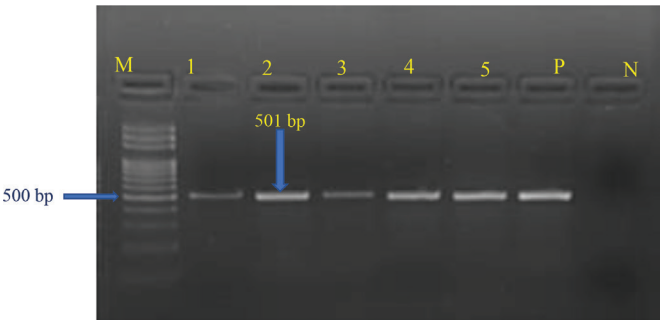

Fig. 2. Agarose gel showing amplified product for *papC* gene of *E. coli* isolates (approx. 501bp)
M: DNA ladder – 1000 bp 1-5: Isolates (Samples)
P: Positive control N: Negative control

The product of *iucD* gene is siderophore (iron binding molecule) aerobactin which enables the microorganism to deprive the host of essential iron, and it helps in the growth and multiplication of bacteria. Without siderophores bacteria would be unable to survive in the host because concentrations of freely available iron in body fluids of

animals and humans are very low (Jansen *et al.,* 2003). The result of present study on *iucD* gene frequency (60.00%) is in the agreement with 65.35% reported by Paixao *et al*. (2016). On the contrary, Oh *et al*. (2011), Arabi *et al*. (2013) and Subedi *et al*. (2018) detected *iucD* gene in tested isolates @ 100%, 85.71% and 97.80%, respectively.

The another siderophore yersiniabactin is encoded by *irp2* gene of APEC helps in similar manner to that of the siderophore aerobactin (Jansen *et al.,* 2003). The present result of *irp2* gene frequency is 46.67%. Subedi *et al*. (2018) and Arabi *et al*. (2013), detected this gene higher than the present study @ 73.30%. and @ 80.51%, respectively, while Roseliza *et al*. (2017) found lower prevalence of *irp2* gene in their study @ 11.20%.

The *astA* gene encodes for EAST1 (enteroaggregative *E. coli* heat-stable enterotoxin 1), which was first observed in EAEC (enteroaggregative *E. coli*) strains, which had been recognized as an agent of diarrhoea (Kwon *et al.,* 2008). The result of *astA* gene frequency (40.00%) in present study is in the agreement with Arabi *et al*. (2013), and Subedi *et al*. (2018), who detected it as 85.71%, and 95.60%, respectively. In contrast, Samanta *et al*. (2013), Paixao *et al*. (2016), and Roseliza *et al*. (2017) detected lower prevalence of *astA* gene @ 14.32%, 23.62%, and 13.60%, respectively.

The *tsh* gene encodes a 140 kDa serine protease present in bacterial periplasm and contain two subunits an extracellular 33 kDa peptide as small subunit and 106 kDa peptide as large subunit (Rocha *et al.,* 2008). The large subunit plays the most important role in bacterial adhesion to host cell during membrane binding process. This subunit can bind to red blood cells, haemoglobin, fibronectin, extracellular matrix proteins and IV collagen. The finding of present study for *tsh* gene frequency (33.33%) concurred with the observations of Paixao *et al*. (2016) and Roseliza *et al*. (2017) as they detected *tsh* gene 30.70% and 32.80%, respectively. Subedi *et al*. (2018) detected moderate prevalence of this gene in their study @ 62.20%. On the contrary, Oh *et al*. (2011) and Ammar *et al*. (2015) detected higher prevalence of tested gene as 82.00%.

Another gene named *vat* encodes a carrier protein that is responsible for vacuolating and transferring of cytotoxin produced by pathogenic strains. Vacuolating cytotoxin is a toxin floated on the APEC surface with a fatal property (Kwon *et al.,* 2008). The frequency of *vat* gene found (26.67%) is in the agreement of Paixao *et al*. (2016) and Roseliza *et al*. (2017) as they detected *vat* gene @ 25.98%, and 16.00%, respectively. In contrast Oh *et al*. (2011) and Arabi *et al*. (2013) detected a high prevalence of *vat* gene @ 94.10% and 85.71%, respectively.

The adhesion factor is P fimbria that is coded by *papC* operon in bacterial chromosome. This factor contributes in prevention of APEC strain to be phagocytosed and it helps in bacterial colonization in respiratory epithelium, which directly affects the intensity of infection (Jansen *et al.,* 2003; Rocha *et al.,* 2008). The findings of *papC* gene frequency (15.00%) is in the agreement of Paixao *et al*. (2016), and Roseliza *et al*. (2017) as they detected *papC* gene at 14.96%, and 9.60%, respectively. Subedi *et al*. (2018) also detected *papC* gene @55.60%. On the contrary, Arabi *et al*. (2013) and Ammar *et al*. (2015) detected high 82% prevalence of *papC* gene in their studies.

In the present study, the APEC-associated genes *iss* and *iucD* were significantly more prevalent (93.34% and 60.00%, respectively) than *irp2* (46.67%), *astA* (40%), *tsh* (33.33%), *vat* (26.67%) and *papC* (15.00%) (Table 1). The least commonly found virulence associated gene was *papC*, which was found in only 9 (15.00%) isolates while most commonly found VAGs in more than 50% isolates were *iss* and *iucD* with 93.34% and 60.00%, respectively. As the prevalence of *iss* and *iucD* genes are more in the isolates indicates that the products of these genes help the APEC to remain protected due to inhibition of the complement system of the host and proliferated, and siderophore aerobactin increase the viability of the organisms in low concentration of freely available iron in the host. The products of other VAGs are useful for APEC by various other mechanisms like iron acquisition, toxin production, adhesion to the host cell, carrier protein for cytotoxin and production of fimbrae. Together all these virulence factors enhance the pathogenicity of the APEC.

Conclusions

The present study involving 60 Avian Pathogenic *E. coli* isolates from various poultry farms revealed that all the isolates possessed at least one virulence associated gene. Among the 60 isolates, two isolates possessed all 7 VAGs, 3 isolates possessed six, 8 isolates possessed five, 9 isolates possessed four, 17 isolates possessed three, 9 isolates possessed two and 12 isolates possessed one VAGs. Among the seven different VAGs, the most commonly found genes in more than 50% isolates were *iss* and *iucD* with 93.34% and 60.00% prevalence, respectively, and *papC* (15.00%) was found less frequent as compared to other genes.

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