RESEARCH ARTICLE

Pentaplex PCR for Rapid and Reliable Detection of Virulence-Associated Genes in Avian Pathogenic *Escherichia Coli* Isolated from Colibacillosis

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Abstract

The present study was conducted with the aim to characterize Avian Pathogenic *Escherichia coli* (APEC) isolates from colibacillosis affected chickens and to screen for key virulence-associated genes using a pentaplex PCR assay. A total of 100 tissue samples were collected from chickens diagnosed with colibacillosis during post-mortem examination. Out of these, 77 samples were presumptively identified as *E. coli* based on cultural and biochemical characteristics. All 77 isolates provisionally identified as *E. coli* were subsequently confirmed by PCR amplification using genus specific primers targeting the 16S rRNA gene. Further screening of the isolates by pentaplex PCR targeting five APEC-associated virulence genes (*iutA*, *iroN*, *iss*, *ompT* and *hlyF*) revealed a high prevalence of these virulence determinants among the *E. coli* isolates obtained from diseased poultry. The *iss* and *ompT* genes were most frequently detected (97.40%), closely followed by *iroN* and *hlyF* (96.10%) and *iutA* (88.31%). Among the isolates, 66 (85.71%) carried all five virulence genes, 9 (11.69%) harbored four genes in various combinations, and only 2 (2.60%) lacked all five genes. These findings substantiate the utility of pentaplex PCR as a rapid, specific, and reliable molecular assay for early identification and differentiation of APEC, thereby supporting prompt intervention and effective control of colibacillosis in poultry production systems.

Key words: APEC, Colibacillosis, Pentaplex PCR, Poultry, Virulence-associated genes.

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Introduction

The poultry industry in India is rapidly growing, contributing significantly to food security, economic growth and sustainable development. It provides affordable, nutritious food to meet rising domestic and global demand, generates employment and income for farmers, and strengthens rural economies. Today, India stands as a major contributor to the global poultry market. However, from time to time, the advancement of poultry production is severely hampered by various infectious diseases (Alam *et al*., 2020). Among these, the colibacillosis caused by Avian Pathogenic *Escherichia coli* (APEC) is a prevalent disease responsible for significant morbidity, high mortality rates, and substantial economic losses in the poultry industry worldwide. It can affect birds of any age, but young birds and developing embryos are more commonly infected and tend to experience more severe symptoms than older birds (Johnson *et al*., 2001). Avian colibacillosis is a localized or systemic infection, characterized by various syndromes including respiratory disease, septicemia, swollen head syndrome, yolk sac infection, omphalitis, and cellulitis, or a combination of these syndromes (De Carli *et al*., 2015). APEC possesses a diverse array of virulence-associated genes that contribute to their pathogenicity. Among these, the plasmid-borne genes *iroN, ompT, hlyF, iss*, and *iutA* have been identified as minimal predictors essential for the characterization of APEC strains (Johnson *et al*., 2008; Ahmed *et al*., 2013).

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The genes *iutA* and *iroN* play a key role in iron acquisition system in APEC isolates (Varga *et al*., 2018). These genes aid in adhesion, invasion, colonization, persistence, virulence gene expression, uptake of iron and manganese from the host, and resistance to environmental stresses (Guabiraba and Schouler, 2015). The *iss* and *ompT* genes encode for protectins which protect bacteria from the host immune system as well as various unfavorable conditions. Protectins include bacterial capsules, outer

membrane proteins, and lipopolysaccharide (LPS) components, and they offer protection against phagocytic engulfment by macrophages as well as complement-mediated bactericidal effect in the host serum (Sarowska *et al*., 2019). The *hlyF* gene encodes for the virulence factor toxins which are biological poisons that helps in the bacterial ability to invade and cause damage to the tissues (Dziva and Stevens, 2008; Sarowska *et al*., 2019). These toxins also facilitate the colonization, motility, biofilm formation, agglutination, induction of vacuolization, and formation of outer membrane vesicles (Murase *et al*., 2016).

These genes are closely linked to the virulence potential of APEC and serve as reliable molecular markers for distinguishing pathogenic strains from non-pathogenic avian fecal *E. coli*. The development and standardization of a pentaplex PCR assay targeting these five key virulence genes provide a rapid, sensitive, and specific diagnostic tool to facilitate early detection and differentiation of APEC, which is crucial for effective disease management and control in poultry production. Hence, this study was undertaken to validate the standardized pentaplex PCR protocol for simultaneous amplification of key APEC virulence markers, enabling early diagnosis, molecular differentiation, and effective disease surveillance to support colibacillosis control strategies in poultry farms.

Materials and Methods

Samples Collection and Isolation

A total of 100 samples were collected from different local poultry farms located in and around Junagadh, Gujarat. The samples included heart (n = 34), liver (n = 33), and spleen (n = 33) obtained from both broiler and layer birds aged between 10 and 80 days. Birds exhibiting lesions characteristic of colibacillosis, such as fibrinous pericarditis, perihepatitis, and airsacculitis, were selected during post-mortem examinations (Fig. 1). Sampling was carried out using sterile instruments and standard aseptic techniques as described by Apostolakos *et al*. (2021), ensuring prevention of cross-contamination.

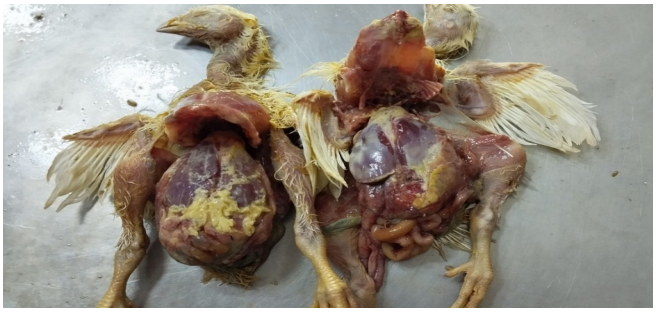

Fig. 1: Deposition of fibrin in heart and liver observed during post-mortem examination of colibacillosis affected chicks

Samples were cultured on brain heart infusion (BHI) agar and incubated at 37°C for 24 h. Gram staining and KOH string tests were carried out to differentiate Gram-positive and Gram-negative bacteria. The colonies identified as Gram-negative bacilli were further sub-cultured on MacConkey agar and Eosin Methylene Blue (EMB) agar, where the production of pink- coloured colonies and typical greenish metallic sheen colonies, respectively, were presumptively identified as *Escherichia coli*. The biochemical characterization was carried out as per the standard procedures (Collee *et al*., 2008) to further confirm the identity of the isolates. Final confirmation of the bacterial identity was achieved by amplification of the genus-specific 16S rRNA gene, following the protocol described by Schippa *et al*. (2010). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 43300 were used as positive and negative controls, respectively.

DNA Extraction

Extraction of bacterial genomic DNA from pure *E. coli* cultures was obtained using heat lysis/snap chill method (Englen and Kelley, 2000). The purity and concentration of extracted DNA was assessed using µDrop™ Plate in µDrop plate reader (Thermo Scientific).

Screening of APEC Virulence Genes by Pentaplex PCR

A total of five virulence genes, *viz., iutA, iroN, iss, ompT* and *hlyF* were targeted using pentaplex PCR assay as previously described by Johnson *et al*. (2008). Primers used for pentaplex PCR are listed in Table 1. The reaction components used for

Table 1: Oligonucleotide primer sets for detection of virulence associated genes in APEC isolates

Sr. No.	Target gene	Description	Primer sequence (5'-3')	Product size (bp)
1	*iutA*	Aerobactin siderophore receptor gene	F: GGCTGGACATCATGGGAACTGG R: CGTCGGGAACGGGTAGAATCG	302
2	*iroN*	Salmochelin siderophore receptor gene	F: AATCCGGCAAAGAGACGAACCGCCT R: GTTCGGGCAACCCCTGCTTTGACTTT	553
3	*iss*	Increased serum survival gene	F: CAGCAACCCGAACCACTTGATG R: AGCATTGCCAGAGCGGCAGAA	323
4	*ompT*	Outer membrane protease gene	F: TCATCCCGGAAGCCTCCCTCACTACTAT R: TAGCGTTTGCTGCACTGGCTTCTGATAC	496
5	*hlyF*	Putative avian hemolysin gene	F: GGCCACAGTCGTTTAGGGTGCTTACC R: GGCGGTTTAGGCATTCCGATACTCAG	450

pentaplex PCR were 12.5 mL of 2X Dream Taq Green PCR Master mix (Thermo Scientific, Lithuania), 0.5 mL pooled forward primer (10 pmoles each), 0.5 mL pooled reverse primer (10 pmoles each), 4.5 mL nuclease free water and 3 mL DNA template. The pentaplex PCR thermal cycling conditions used were initial denaturation at 94° C for 2 min, followed by 25 repeat cycles of denaturation at 94° C for 30 sec, annealing at 62° C for 30 sec, extension at 67.5° C for 3 min, and final extension at 72°C for 10 min. To confirm the target amplification, 5 mL of PCR product was mixed with 6X DNA loading dye and electrophoresed on a 1.5% agarose gel alongside a 100 bp Plus DNA Ladder (GeneRuler®, Thermo Scientific) at 5 V/cm until the tracking dye migrated out of the gel. The amplified product appeared as a single band of expected size under UV light and was documented using a Bio-PrintST4® gel documentation system (Vilber Lourmat).

Results and Discussion

Out of 100 samples examined, *E. coli* was isolated from 77 (77%) samples, based on colony morphology and biochemical characteristics. All isolates were catalase positive, oxidase negative, indole and methyl red positive, and Voges-Proskauer negative, consistent with the typical *E. coli* IMViC profile (+, +, –, –). Most of the isolates were citrate negative, while 15 (19.48%) isolates were citrate positive, indicating citrate utilization as a sole carbon source. Among the different organs examined, similar prevalence of *E. coli* was detected in all three organs, with rates of 33.76% each for heart and liver and 32.48% for spleen. Our findings were in close agreement with those of Levy *et al*. (2020) and Menck-Costa *et al*. (2023), who reported prevalence rates of *E. coli* associated with colibacillosis of 82.8% and 73.0%, respectively. Likewise, relatively higher prevalence rates (91.0% and 100%) were reported by Ahmed *et al*. (2013) and Subedi *et al*. (2018), respectively. A comparatively lower prevalence rates of 36.5% and 37.0% of *E. coli* were observed by Younis *et al*. (2017) and Tawakol and Younis (2019) from colibacillosis-affected poultry farms. A lower prevalence rate of only 6.32% *E. coli* isolates was observed by Tongkamsai and Nakbubpa (2024). The variation in prevalence rates reported across different studies may be attributed to factors such as differences in sample type, bird health status (apparently healthy birds versus those with suspected colibacillosis), geographic location, farm level biosecurity practices, and laboratory methodologies, all of which play crucial roles in influencing the reported isolation rates.

In the present study, age-wise stratification revealed that the prevalence of *E. coli* infection was highest in birds aged between 0-15 days (46.76%), followed by 31-45 days (27.27%) and 16-30 days (25.97%), while no cases were detected beyond 46 days of age. This finding suggests that susceptibility to *E. coli* is markedly higher in younger birds, which may be attributed to an immature immune system, higher physiological stress, and increased vulnerability during the early stages of growth. Similar finding was reported by Panchal *et al*. (2020), who documented a higher prevalence in younger age groups of broiler birds compared to older birds, emphasizing age as a critical risk factor in colibacillosis.

All bacterial isolates were exclusively recovered from broiler birds (100%), while no bacterial isolates were obtained from layer birds (0%). This variation may be explained by differences in genetic makeup, growth rates, stocking densities, and management practices between broilers and layers. Broilers, being fast-growing and reared under intensive conditions, are often more susceptible to stress and opportunistic infections. These findings are consistent with reports by Panchal *et al*. (2020) and Veloo *et al*. (2025), who demonstrated higher prevalence of *E. coli* in broiler farms relative to layer or indigenous farms, highlighting the significant impact of production system differences on the epidemiology of avian colibacillosis. Contrary to our findings, Rahman *et al*. (2004) reported high prevalence of *E. coli* infection in layer birds (36.73%) compared to broilers birds (2.52%).

The expected 585 bp amplicon of the 16S rRNA gene was successfully amplified in all 77 isolates, confirming their identity as *E. coli* (Fig. 2). The findings of the present study aligned with those of Parvin *et al*. (2020) and Jhandai *et al*. (2025), who also reported that 100% of their isolates were molecularly confirmed as *Escherichia coli* via PCR amplification targeting the 16S rRNA gene. This consistent result across studies demonstrates the reliability of 16S rRNA gene-based PCR for accurate identification of *E. coli* isolates.

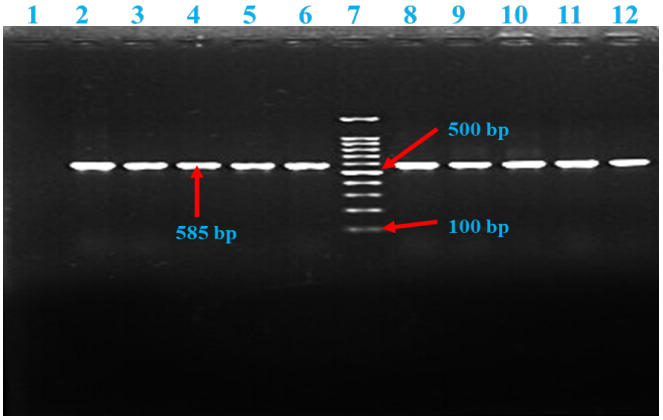

Fig. 2: Agarose gel electrophoresis of genus specific 16S rRNA gene (585 bp). Lane 1= Negative control (*Staphylococcus aureus* ATCC 43300), Lane 2 to 5 and 8 to 12= positive samples, Lane 6= Positive control (*Escherichia coli* ATCC 25922), Lane 7= 100 bp plus DNA ladder

The pentaplex PCR assay developed in this study targeting the five minimal APEC virulence genes *(iutA*, *iroN, iss, ompT and hlyF*) demonstrated to be a rapid and specific diagnostic tool for the identification and characterization of APEC isolates. The targeted virulence-associated genes were detected at high frequencies, with *iss* and *ompT* being the most prevalent (97.40%), followed by *iroN* and *hlyF* (96.10%) and *iutA* (88.31%)(Table 2). Overall, the present study revealed that 66 (85.71%) isolates possessed all five virulence associated genes, 9 (11.69%) isolates possessed four genes

Table 2: Genotypic detection and distribution of virulence associated genes among *E. coli* isolates from colibacillosis affected chicken (n=77)

Name of Virulence associated genes		*iutA*	*iroN*	*iss*	*ompT*	*hlyF*
No. (%) of positive isolates		68 (88.31)	74 (96.10)	75 (97.40)	75 (97.40)	74 (96.10)
No. (%) isolates positive for Virulence associated genes	5 genes			66 (85.71)		
	4 genes			9 (11.69)		
	No genes			2 (2.60)		

of different combinations, while 2 (2.60%) isolates did not possessed any of the five virulence associated genes.

The agarose gel electrophoresis revealed that amplification products of the gene, *iss* (323 bp) and *iutA* (302 bp) showed no distinct separation due to the close proximity of their product sizes, resulting in a thick merged band. However, the products for *iroN, ompT*, and *hlyF* genes could be distinctly identified owing to the significant differences in their amplicon sizes. Additionally, a non-APEC isolate in lane 9 was clearly differentiated from the APEC isolates (Fig. 3).

Similar to our findings, a high presence of these genes has been consistently reported by various scientists. Subedi *et al*. (2018) reported 100% detection of *iss, ompT, hlyF* and *iroN* genes among 45 APEC isolates, with *iutA* being highly frequent (82.2%). Pilati *et al*. (2024) found *iss* and *ompT* genes in 96.5% each, *hlyF* in 93.1%, *iutA* in 94.8% and *iroN* in 89.6% isolates, which closely parallels the current results. Likewise, Grakh *et al*. (2022) detected all five genes in 100% of isolates, except *iss* (95.7%). The concordance of these results with the present study reinforces the idea that these five genes are strongly conserved among pathogenic strains of Avian Pathogenic *E. coli*.

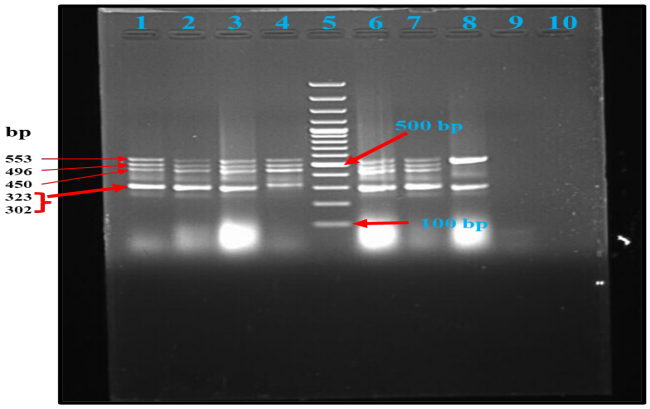

Fig. 3: Agarose gel electrophoresis of pentaplex PCR showing amplified product of *iutA* (302 bp), *iroN* (553 bp), *iss* (323 bp), *ompT* (496 bp) and *hlyF*(450 bp) genes. Lane 1 to 3 and 6 to 7= APEC positive isolates (five genes), Lane 4=APEC positive isolate (four genes), Lane 5=100 bp DNA ladder; Lane 8= *Escherichia coli* ATCC 25922 (genus specific), Lane 9=non-APEC isolate.

In contrast, some earlier studies documented considerable lower detection rates of these virulence markers. Li *et al*. (2015) recorded *iroN* gene in 64.4%, *iutA* gene in 60.9%, *iss* gene in 42.5%, *hlyF* gene in 35.6% and *ompT* gene in only 5.7% of isolates. Radwan *et al*. (2014) also reported a low prevalence of *iutA* (5%) despite high detection rates of *iroN, ompT*, and *hlyF*, each present in 80% of the isolates. Similarly, Mbanga and Nyararai (2015) found *iutA* in 80% of isolates, but *hlyF* and *ompT* were present in only 24.4% and 2.2%, respectively. These figures demonstrate a wide range of variability compared to the near-universal prevalence observed in the present investigation. Such discrepancies may be explained by differences in geographical distribution of isolates, host factors, sample types, or variations in PCR assays used.

Conclusions

Given the growing concern over APEC as a poultry pathogen with zoonotic potential, the pentaplex PCR serves as a promising molecular tool for routine use in clinical and epidemiological surveillance. By simultaneously amplifying five plasmid-borne virulence genes, this assay effectively captures the molecular markers that define the APEC pathotype, distinguishing pathogenic strains from commensal or non-pathogenic avian *E. coli*. Its efficiency, affordability, and rapid turnaround overcome the limitations of conventional serotyping and phylogenetic typing, enabling timely diagnosis and targeted interventions. Overall, the pentaplex PCR holds strong potential to strengthen disease monitoring, minimize economic losses, and mitigate public health risks associated with avian colibacillosis in poultry production systems.

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