*The Haryana Veterinarian* (June, 2023) **62(1),** 19-22 ISSN 0033-4359 (P) **Research Article**

# STANDARDIZATION OF PENTAPLEX PCR FOR RAPID DIAGNOSIS OF AVIAN PATHOGENIC *ESCHERICHIA COLI* INFECTION

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## ABSTRACT

Avian colibacillosis is one of the leading causes of mortality and morbidity in poultry industry worldwide including India. The causative agent of avian colibacillosis is Avian pathogenic *E. coli* (APEC), which is nowadays characterized by amplification of different virulence genes using polymerase chain reaction (PCR). To amplify sets of different virulence genes using individual gene amplification-based PCR is a tedious, slow and costly procedure especially for outbreak samples. For timely and effective control and preventive measures, the diagnosis needs to be rapid, simple and dependable in terms of sensitivity and specificity. For this purpose,we used previously characterized APEC isolates and standardized the pentaplex PCR assay using optimized conditions. The assessment of sensitivity and specificity was done, and it was found that 200 ng of template DNA was enough to amplify fragments of all the five genes (*hlyF*, *ompT*, *iroN*, *iss* and *iutA*) used in the study. The assay was found to be highly specific for the detection of primer specific amplification of targeted genes of APEC and only single target amplification (*iutA*) was seen for Urinary pathogenic *E. coli* (UPEC) of canine origin which is a subtype of Extraintestinal pathogenic *E. coli* (ExPEC). Therefore, the current study suggests that pentaplex PCR assay is more simple, rapid, sensitive and specificto diagnose and characterize APEC, and for epidemiological studies.

**Keywords:** Avian pathogenic *E. coli*, Pentaplex PCR, Sensitivity, Specificity, Virulence genes

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Avian pathogenic *E. coli* (APEC) is a subtype of Extra-intestinal pathogenic *E. coli* (ExPEC) which causes extra intestinal infections in poultry birds. As a primary pathogen of poultry birds APEC is responsible for localized as well as systemic lesions in birds which are collectively termed as avian colibacillosis (Dziva and Stevens, 2008). APEC is normally found in the intestinal tract of birds along with non-virulent strains and adopt to extra intestinal translocation only in presence of stressors (Leitner and Heller, 1992). The avian colibacillosisis a constant occurrence at poultry farms especially commercial broiler farms (Grakh *et al.*, 2020). The huge genetic diversity among APEC makes it particularly challenging to diagnose, prevent and treat avian colibacillosis. Traditional methods of diagnosis for avian colibacillosis are clinical signs, pathology, culture of APEC from the infected lesions and serogrouping (Nolan *et al.*, 2013). However, these methods are nonspecific and time consuming when compared with molecular detection of APEC from field samples. Several studies, emphasize on the finding that the presence of a specific serogroup does not reflect the pathogenicity of APEC strains (Delicato *et al.*, 2003; Ewers *et al.*, 2007). Moreover, overlapping of APEC serogroups with commensal *E. coli* strains and identification of a greater number of newer untypable serogroups of APEC have reduced significance of serogroup as a diagnostic tool (Ewers *et al.*, 2007; Schouler *et al.*, 2012; Kumar *et al.*, 2018). Similarly, overlapping of phylogroups within pathogenic and non-pathogenic isolates makes it tedious and less reliable procedure (Mittal *et al.*, 2022). Molecular methods have proven to be reliable, cost-effective, sensitive, specific and less tedious than conventional culture methods (Garofalo *et al.*, 2007). Several studies have suggested that the prevalence of certain virulence genes (VGs) among *E. coli* isolates obtained from chickens with colibacillosis are useful to identify and characterize APEC and distinguish it from commensal *E. coli* (Johnson *et al.*, 2008). The geographical variations in the prevalence of APEC-associated VGs also reflect the vital role of defining APEC on the basis of VGs from indigenous commercial broiler chickens. The set of five virulence genes (*hlyF*, *ompT*, *iroN*, *iss* and *iutA*) for characterization and differentiation have been used previously across the world (Johnson *et al.*, 2008). Although in our previous study (Grakh *et al.*, 2022) characterization of APEC and their differentiation from commensal *E. coli* isolates was done using a set of five virulence genes employing individual target amplification where individual PCR reaction consumed a lot of time hampering rapid results and utilization of more resources and time. This study was thus designed to timely diagnose the avian colibacillosis by standardizing a pentaplex PCR assay and to save the cost associated with diagnosis.

## MATERIAL AND METHODS

### Source of samples

The sample collected and analyzed for various traits

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such as antibiotic susceptibility, virulence genotyping and biofilm formation in previous study by Grakh *et al.* (2022) were used for the standardization of pentaplex PCR. A total of eight APECand one non-APEC isolate were used for the purpose (Grakh *et al.*, 2022; Mittal *et al.*, 2022).

**DNA extraction**

The genomic DNA extraction from the cultured colonies was obatined using heat lysis/snap chill method (Englen and Kelley, 2000). The concentration of DNAwas measured using Bio Photometer D30 (Eppendorf, Germany).

**Standardization of pentaplex PCR**

A total of five genes were targeted using primer pairs designed as per literature available (Table 1). The reaction components used for pentaplex PCR were Amp fast master mix (2x) (Takara) 12.5 µl, pooled forward primer (10 µM each), pooled reverse primer (10 µM each), nuclease free water 5 µl and DNA template at a concentration of 400 ng to 25 ng per 25 µl reaction. The pentaplex PCR thermal cycler conditions were initial denaturation at 94° C for 2 min; with 25 cycles of denaturation at 94° C for 30 s, annealing at 62° C for 30 s, extension at 67.5° C for 3 min; and a final cycle of extension at 72° C for 10 min followed by a hold at 4° C. The DNA from the APEC positive isolate APEC41LFB (GenBank Accession number: OL457183, OL457184, OL457185, OL457186, OL457187 for five genes) (Grakh *et al.*, 2022) was used as a positive control in current study. Atotal of eight APEC isolates and one non-APEC isolate were used in the study along with a quality control containing distilled water as template. The pentaplex PCR was repeated three times with same samples to reconfirm the presence of genes.

**Sensitivity of the pentaplex PCR**

The sensitivity was determined using diluted DNA templates with two-fold dilutions from 400 ng in nuclease free water. The PCR was performed under same optimized conditions as described above. A total of ten reactions with 400 ng; 200 ng; 100 ng; 50 ng and 25 ng were prepared in duplicate using two APEC positive isolates for sensitivity analysis of pentaplex PCR. The nuclease free water was adjusted accordingly in a 25 µl PCR reaction.The reaction volume was kept at 25 µl to ensure cost effective method as per the protocol used by Wang *et al.* (2014). The *E. coli* isolates were characterized as APEC and non-APEC as per the criterion described in the literature (Johnson *et al.*, 2008; Grakh *et al.*, 2022).

**Specificity of the pentaplex PCR**

For the analysis of specificity of pentaplex PCR, DNA from *E. coli* reference strain (ATCC 25922), two Urinary pathogenic *E. coli* (UPEC; available in the department) of canine origin and *Staphylococcus aureus* was used as the template for the PCR reaction. The negative control contained distilled water instead of DNA.

**Agarose gel electrophoresis**

Amplified PCR products were analyzed by agarose gel electrophoresis using 2% concentration of agarose in tris acetate EDTA buffer (TAE) stained with 0.5 µl/ml of ethidium bromide at a constant 80 V. A molecular marker of 100 bp (Takara, Japan) was used to compare the PCR products obtained. Amplified products in the gel were digitized using UV transillumination in a gel doc system.

## RESULTS AND DISCUSSION

The conventional diagnostic methods for avian colibacillosis and APEC, based on serotype and phylogenetic grouping are not much specific due to diversity and overlapping with non-pathogenic *E. coli* isolates (Schouler *et al.*, 2012). Therefore, for accurate diagnosis of such infections, characterization of APEC based on the presence of virulence genes was used by various researchers around the world. As geographical variations in terms of prevalence of virulence genes exists (Hussein *et al.*, 2013; Dissanayake *et al.*, 2014), PCR standardization for individual gene was done previouslyby Grakh *et al.* (2022) in India to establish the genes as predictor for APEC. As the genes used for standardization are mostly present on Col V plasmid of APEC and are considered conserved, all the serotypes and phylogenetic groups ofAPEC can be diagnosed effectively by this pentaplex PCR.

**Standardization of pentaplex PCR assay**

The isolates used in the study were previously characterized as APEC based on the individual fragment amplification by conventional PCR. The standardization of pentaplex PCR was done using DNA template of APEC positive isolates at 400 ng per reaction. The results obtained on gel electrophoresis of amplified products as shown (Fig. 1) can be appreciated with five distinct bands visible. There is less demarcation of amplified fragments of *iss* and *iutA* genes and this is due to less difference in their product size i.e., 323 bp and 302 bp, respectively. Despite such closeness in the size two different bands are visible. The products obtained for *iroN*, *ompT* and *hlyF* can be distinguished easily due to appreciable difference in amplified product size. Anon-APEC isolate in lane 9 (Fig. 1) is also well appreciated to differentiate it from APEC isolates. The standardization of pentaplex PCR thus efficiently confirmed the presence of all the genes which proves its usefulness in rapiddiagnosis ofAPECaswellas differentiation from non-APEC *E. coli* isolates. Use of multiplex PCRs for detection and confirmation of pathogens provides a quick and reliable tool which is much needed for diseases with outbreak potential (Wang *et al.*, 2014). Additionally, use of

**Table 1. Primer pairs used for standardization of pentaplex PCR for APEC detection**

| Sr. No. | Description | Gene | Primers (5'-3') | Product size (bp) |
|---|---|---|---|---|
| 1. | Salmochelin siderophore receptor gene | *iroN* | F- AATCCGGCAAAGAGACGAACCGCCT<br>R- GTTCGGGCAACCCCTGCTTTGACTTT | 553 |
| 2. | Episomal outer membrane protease gene | *ompT* | F-TCATCCCGGAAGCCTCCCTCACTACTAT<br>R-TAGCGTTTGCTGCACTGGCTTCTGATAC | 496 |
| 3. | Putative avian Hemolysin gene | *hlyF* | F-GGCCACAGTCGTTTAGGGTGCTTACC<br>R-GGCGGTTTAGGCATTCCGATACTCAG | 450 |
| 4. | Episomal increased serum survival gene | *iss* | F-CAGCAACCCGAACCACTTGATG<br>R-AGCATTGCCAGAGCGGCAGAA | 323 |
| 5. | Aerobactinsiderophore receptor gene | *iutA* | F-GGCTGGACATCATGGGAACTGG<br>R-CGTCGGGAACGGGTAGAATCG | 302 |

**Source:** Johnson *et al.* (2008)

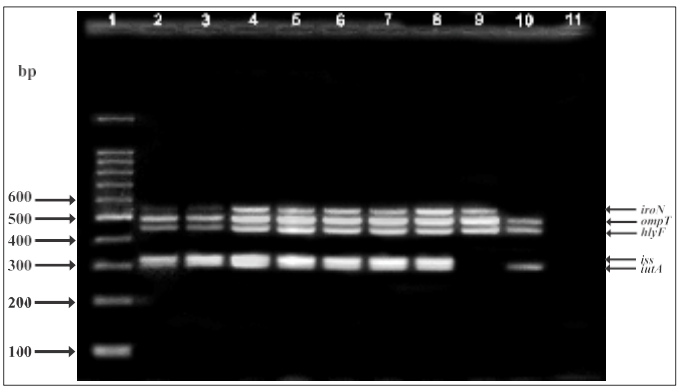


Fig. 1. Agarose gel electrophoresis of pentaplex PCR showing amplified product of *iroN*, *ompT*, *hlyF*, *iutA* and *iss* genes, lane 1-100 bp DNA ladder; Lane 2 to 8 APEC positive isolates (five genes), lane 10 – APEC positive isolate (four genes), lane 9-non-APEC isolate (3 genes), lane 11–negative control

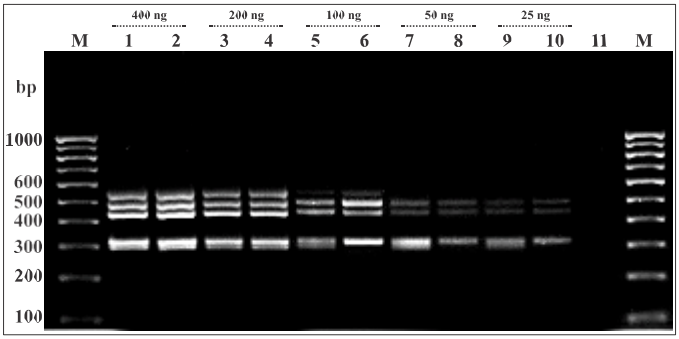


Fig. 2. The sensitivity analysis of the pentaplex PCR assay. The detection limit was 100 ng of bacterial DNA. Lane M: 100 bp DNA marker, Lane 1 and 2: APEC isolates with DNA template concentration of 400 ng, Lane 3 and 4: DNA template concentration of 200 ng, Lane 5 and 6: DNA template concentration of 100 ng, Lane 7 and 8: DNA template concentration of 50 ng, Lane 9 and 10: DNAtemplate concentration of 25 ng, Lane 11: negative control.

pentaplex PCR will save on the cost incurred in confirming the presence of individual gene using conventional PCR. The DNA extracted from tissue samples, water, litter, and other materials from avian colibacillosis affected poultry farms can be directly used to diagnose the cause i.e., APEC.

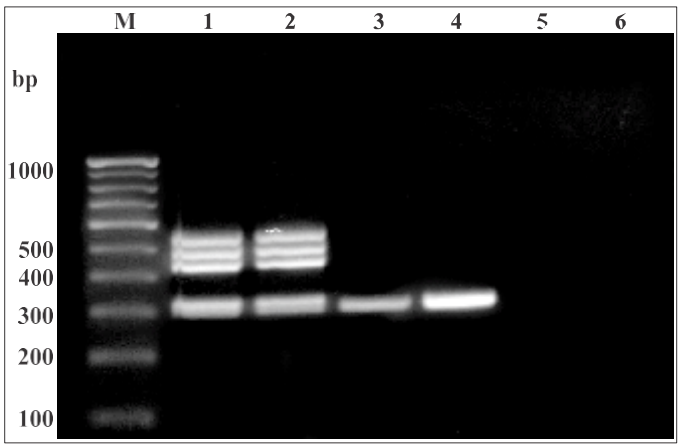


Fig. 3. The product profile ofAPEC and UPEC isolates used for specificity assessment of pentaplex PCR assay. Lane M: 100 bp DNA marker, Lane 1 and 2: APEC isolates positive for all five genes, Lane 3 and 4: UPEC isolates positive for *iutA*, Lane 5: *Staphylococcus aureus* (negative), Lane 6: Negative control

### Assessment of the sensitivity of the pentaplex PCR assay

However, standardization of pentaplex PCR enabled quick diagnosis and characterization of APEC, its sensitivity and specificity assessment were also done to decide its implication and suitability for its use a diagnostic tool. The sensitivity of the assay was determined using a series of diluted bacterial DNA concentration. The results revealed that this pentaplex PCR can be effectively used for 400 ng and 200 ng concentration of template (Fig. 2). All the five amplified products at these concentrations can be easily appreciated and demarcated. Both the APEC isolates used yielded sharp bands for all the five genes used for characterization. At 100 ng however all the amplified products can be visualized but the faint band for one gene (*iutA*) might limit its sensitivity. However sharp and well demarcated bands are observed at 400 and 200 ng but if less DNA is available, 100 ng can also be used as presence of four genes also clustered the isolates to APEC. So, for detection of APEC using pentaplex PCR with high

sensitivity a DNA template of concentration more than 200 ng is sufficient and can be used effectively. The amplified products at 50 ng and 25 ng are neither well demarcated nor sharp, so these concentrations should not be used for detection of APEC. The lower sensitivity of multiplex PCR assays might be because of possible interferences among the primers in the reaction (Wang *et al.*, 2014). The pentaplex PCR assay was successful in characterizing and differentiation from non-APEC of poultry origin as well as canine origin and other bacterial species.

**Assessment of the specificity of the pentaplex PCR assay**

In order to evaluate the specificity of the primers used in this study, PCR were performed using different bacterial templates. The DNA from two APEC, two UPEC of canine origin and one *Staphylococcus aureus* was used as the template for the PCR reaction. The results showed that particular gene specific fragments were amplified using respective primers for five genes as mentioned above (Table 1). No fragment was amplified from *Staphylococcus aureus* template. However, one fragment (*iutA*) was amplified in two isolates (UPEC). The amplification of *iutA* gene is justifiable in UPEC, as it is a subtype of ExPEC and these isolates share some of the virulence characteristics with APEC (Huan *et al.*, 2007). The pentaplex PCR standardized in the current study is highly specific for the diagnosis of APEC (Fig. 3) and can be reproduced and used as a diagnostic tool for rapid diagnosis of APEC. These results revealed that pentaplex PCR standardized in current study was sensitive and specific and can be used for simple, rapid, reliable, and cost-effective diagnosis of avian colibacillosis and APEC.

Conclusively, the current study standardized apentaplex PCR assay using a set of five virulence genes characteristic to APEC. This assay was able to differentiate APEC from non-APEC such as UPEC, commensal avian *E. coli* and bacteria of other species such as *Staphylococcus aureus* with high sensitivity and specificity. This PCR assay was an efficient and convenient strategy for rapid diagnosis and characterization of APEC pathotype and might overcome the disadvantages of serotyping and phylogenetic grouping-based assays. Thus, development of this pentaplex PCR assay will benefits the clinical diagnostics, epidemiological studies and control of avian colibacillosis.

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