Characterization and antimicrobial sensitivity of biofilm producing Avian Pathogenic Escherichia coli from broiler chickens and their environment in India.

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Research Article

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Abstract

Avian pathogenic Escherichia coli (APEC) is responsible for colibacillosis in poultry. APEC remains a constant problem for the poultry industry, despite the use of antibiotics and disinfectants at farms. The endemicity of APEC in poultry farms is associated with its biofilm forming ability, which is further aggravated by various virulence factors harbored by this pathogen and resistant to multiple drugs which help bacteria to thrive under different environmental conditions. To characterize APEC from affected broiler chickens and their environments, samples (n=114) were collected and subjected to microbial isolation using MacConkey Lactose agar (MLA) and Eosin Methylene Blue agar (EMB), which led to the isolation of 62 E. coli isolates, confirmed using uspA gene amplification and Vitek 2 Compact system. These isolates were characterized using a set of five virulence genes (hlyF, ompT, iroN, iss, iutA) which yielded 47 (75.80%) isolates as APEC and the remaining as non-APEC. Furthermore, all the 62 isolates were subjected to microtiter plate assay for biofilm detection and the result showed that 36 (58.06%) isolates were able to form moderate to strong biofilms in Trypticase soy broth (TSB) at 72h of incubation. Of the 36-biofilm forming isolates, 30 were APEC. Biofilm related genes (crl, csgA, fimH, luxS, and papC) were also detected with higher prevalence among APEC isolates. Antimicrobial sensitivity test using Vitek2 system revealed 43 (91.48%) of 47 APEC isolates as multiple drug resistant (MDR) and 8 (17.02%) as ESBL positive. This study reveals that APEC with biofilm formation ability are present in poultry farms. Further studies are needed to understand the role of biofilms in the pathogenesis and antimicrobial resistance of APEC.

Introduction

APEC, an extraintestinal pathogen of birds, is an important cause of economic losses to the poultry industry in terms of morbidity, mortality, condemnation of carcasses, decreased FCR, and costs incurred in treatment and disinfection (Graveline et al. 2014). APEC causes systemic to localized lesions in poultry birds including pericarditis, perihepatitis, air sacculitis, salpingitis, yolk sac infection, omphalitis, synovitis, cellulitis, ascites, and chronic respiratory disease (Nolan et al. 2020). The disease occurs in all types and age groups of birds in the presence of stressors or predisposing factors (Grakh et al. 2020). APEC is a subtype of ExPEC (Extra intestinal pathogenic *E. coli*), which causes infections in humans and thus suggests its zoonotic potential.

The mere isolation of *E. coli* from affected birds is not sufficient to establish its pathogenicity as it is a natural commensal/non-pathogenic bacterium living inside the gut of birds known as AFEC (Avian Fecal *E. coli*) and commonly present in the surrounding environment. Thus, there is a need for characterization of APEC and their differentiation from non-APEC/AFEC. Several studies have approached serotyping to characterize APEC, but due to overlapping serogroups of AFEC, serogrouping cannot be defined as a diagnostic tool to address the virulence of *E. coli* (Paixao et al. 2016). Previous studies have suggested that virulence factors are responsible for the pathogenicity of APEC (Dou et al. 2015). Although there are several virulence factors related to the extraintestinal pathogenicity of APEC, hlyF, *ompT*, *iroN*, *iss*, and

iutA carried on colV plasmid are considered as the most significantly associated with APEC strains and are widely used for characterization (Johnson et al. 2008; Dou et al. 2015; Awawdeh, 2018).

Most of the current treatment strategies focus on early prevention of the disease in broiler flocks by using antibacterial drugs rather than treatment after infection, which facilitate antimicrobial resistance (Nolan et al. 2020). Increasing evidence suggests that the transfer and dissemination of drug-resistant strains and genes among human and animal pathogens is possible, which poses a global threat to the poultry industry as well as the public (Aarestrup, 2005; Dou et al. 2015).

Biofilms are complex communities of bacteria which are irreversibly attached to surfaces and are suspended in their own secretions of extracellular substances. Persistent infection, sharing of drug resistant and virulence gene, and resistance to normal doses of antibiotics are few of the advantages conferred by the biofilm to bacteria, all of which makes it difficult to treat such infections (Kester and Fortune, 2014). Biofilm associated genes such *crl*, *csgA*, *fimH*, *luxS*, and *papC* are regarded as important virulence factors for attachment and infection initiation in the host and are previously also linked to antimicrobial resistance (Wang et al. 2016). The *crl* and *csgA* genes encode for curli and responsible for the initial attachment of APEC to host cells or surfaces, *fimH* encodes for the adhesive subunit of F1 fimbriae which facilitates colonization, invasion, and formation of biofilm like intracellular bacterial communities (Schwartz et al. 2013; Cunha et al. 2017). The *luxS* gene mediates the synthesis of AI-2 (auto-inducer) and regulates the motility, biofilm formation, virulence, and pathogenesis of APEC and *papC* genes, which enables the adherence and survival of *E. coli* in the internal organ in canines and humans (Yu et al. 2018).

In the present study, APEC was isolated from broiler chickens and their environment and characterized using a set of five virulence genes previously described in literature. Biofilm formation and quantification and antimicrobial resistance were assayed. APEC and non-APEC isolates were also screened for biofilm related genes to assess their relevance to the biofilm forming ability of these isolates. To the best of our knowledge, this is the first elaborative study in India for the characterization of APEC using pentaplex PCR (five genes) and their biofilm formation on commercial broiler flocks. The findings of this study may act as a base for future studies directed towards the control and prevention of avian colibacillosis.

Materials And Methods

Source of samples

A total of 10 avian colibacillosis affected broiler chicken farms in five districts in the state of Haryana were identified for sample collection based on the criteria of Vandekerchove et al. (2004), i.e., (1) the farmer reported an increase in mortality, compared to normal routine mortality in the flock (2) detection of lesions typical/compatible with colibacillosis after necropsy of three birds; and (3) *E. coli* isolated from heart, liver/lungs in pure or abundant cultures. The samples (n = 114) included: tissue, cloacal swabs, and

poultry farm environment (Table 1). All the samples were collected with consent of poultry farmers. Samples immediately after collection were transported on ice and processed the same day in laboratory.

Table 1
Samples collected from various sources (n = 114)

Source	**Colibacillosis affected birds (n = 32)**		**Poultry farm environment (n = 82)**					
No. of samples	Tissue samples	Cloacal swab	Polyvinyl chloride (PVC) pipe swab	Feeder + Drinker	Tank water	Feed	Litter	Tank wall
	22	10	14	26	12	10	10	10

Isolation and identification of E. coli

Tissue samples were plated directly on MacConkey (MLA) agar. First, the surface of the tissue was sterilized with a hot spatula, the sample was taken by inoculating sterile loops with the tissue and plated on MLA. Feed, litter, and water was processed as described by Samanta et al. (2014). Briefly, 4gm feed + 36ml BHI broth, 4gm litter + 36ml BHI broth, and 5ml water + 45ml BHI broth were incubated at 37°C for overnight incubation. A loopful of BHI broth mixed samples were streaked on MLA using a sterile inoculation loop and incubated for 24h at 37°C. The swab samples of cloaca, PVC pipe, feeder, tank wall and drinker were plated on MLA after dilution in Brain Heart infusion (BHI) broth and incubated at 37°C for 24h. The typical fermentating colonies were selected from MLA plates and were stained by Gram's staining method to determine the presence of other bacterial isolates. The colonies after gram staining were further purified by re-streaking on MLA and plated on EMB agar followed by an incubation of 24h at 37°C. The bacterial colonies showing typical pink to red fermentation on MLA and purple-bluish precipitation on EMB with or without metallic sheen were tentatively considered as *E. coli* and given the isolate numbers accordingly.

These *E. coli* isolates were confirmed using Vitek 2 Compact system. Gram negative (GN) reagent cards were used for identification of *E. coli* as per the recommendation of the manufacturer and the method described previously (Mittal et al. 2018).

PCR amplification for confirmation of E. coli

The genomic DNA extraction from Vitek 2 confirmed colonies was carried out using a heat lysis/snap-chill method (Englen and Kelley 2000). The supernatant was taken and stored as DNA for further use at -20°C. The DNA from ATCC 25922 was used as a positive control for *E. coli*. The PCR amplification was performed using primer pairs for universal stress protein (*uspA* gene) (Osek 2001). The PCR reactions were carried out in 25µl volumes in an Applied Biosystem Veriti thermocycler with the following conditions: 95°C 2 min; 94°C 30 s, 58°C 1 min, 72°C 1 min, total of 30 cycles; 72°C 5 min, hold at 4°C.

Characterization of APEC

PCR primers targeting virulence genes for APEC characterization were used as per the literature (Johnson et al. 2008; Awawdeh 2018) for detection of five virulence genes (Table 2). The PCR was first performed separately for each virulence gene as described by Johnson et al. (2008) using APEC O2 as a positive control available in the department. The *E. coli* isolates harboring more than or equal to four genes were taken as APEC isolates and the isolates harboring less than four or no genes were taken as non-APEC as the criterion described in the literature (Johnson et al. 2008; Awawdeh 2018). The *E. coli* isolates found to be positive for virulence genes individually were then taken as positive controls for standardization of pentaplex PCR for detection of all five virulence genes in a single reaction at the same time. A reaction of 25 µl was used for individuals as well as pentaplex PCR with the following conditions: 94°C 2 min; 94°C 30 s, 63°C 30 s, 68°C 3 min, total 25 cycles; 72°C 10 min, held at 4°C.

Table 2
Primer pairs used for characterization of APEC (Johnson et al. 2008)

Sr. No.	Description	Gene	Primers (5'-3')	Product size (bp)
1	Salmochelin siderophore receptor gene	*iro*N	F- AATCCGGCAAAGAGACGAACCGCCT R- GTTCGGGCAACCCCTGCTTTGACTTT	553
2	Episomal outer membrane protease gene	*omp*T	F-TCATCCCGGAAGCCTCCCTCACTACTAT R-TAGCGTTTGCTGCACTGGCTTCTGATAC	496
3	Putative avian hemolysin	*hly*F	F-GGCCACAGTCGTTTAGGGTGCTTACC R-GGCGGTTTAGGCATTCCGATACTCAG	450
4	Episomal increased serum survival gene	*iss*	F-CAGCAACCCGAACCACTTGATG R-AGCATTGCCAGAGCGGCAGAA	323
5	Aerobactin siderophore receptor gene	*iutA*	F-GGCTGGACATCATGGGAACTGG R-CGTCGGGAACGGGTAGAATCG	302

Agarose gel electrophoresis

Amplified PCR products were analyzed by agarose gel electrophoresis using 2% agarose in tris acetate EDTA buffer (TAE) stained with 0.5 µl/ml of ethidium bromide at a constant 80V. Amplified products in the gel were digitized using UV transillumination in a gel doc system.

Biofilm formation and quantification

Biofilm formation and quantification was done in 96-well polystyrene microtiter plates as described by Skyberg et al. (2007) with minor modifications. Briefly, the APEC and non-APEC colonies were inoculated in 5ml of Luria-Bertani (LB) broth and incubated overnight at 37°C. Turbidity of the broth was adjusted to OD600 = 0.05 by diluting the overnight broth culture in TSB broth. A volume of 200 µl aliquots of the dilution were then dispensed into microtiter plate (Corning, USA) wells in triplicate. Uninoculated TSB media was used as a negative control and ATCC 25922 was used as a positive control for biofilm production (Branco et al. 2016). The plates were inoculated and incubated aerobically without shaking at 37°C for 72h. The content of the plates was gently poured off and the plate was washed with double distilled water and each well was stained with 200 µl of 0.1% crystal violet for 30 min. The plates were washed three times with double distilled water to remove excess stain followed by air drying for 30 minutes. To each well, a 250 µl solution of ethanol: acetone (80:20) was added for solubilizations of adherent cells. A volume of 150 µl of this solution was transferred to a new microtiter plate and OD of each well was measured at 570 nm (OD570) using an automated microplate ELISA reader. The tests were carried out in triplicate and the average value of the results were taken. The cutoff OD (ODc) was calculated as ODc = average OD of negative control + 3 (SD of negative control), where SD is the standard deviation. The isolates were grouped into four grades based on biofilm forming capabilities (Stepanovic et al. 2004).

$OD \leq ODc$ = Strains with no biofilm forming ability

$ODc < OD \leq (2x\ ODc)$ = Strains with weak biofilm forming ability

$(2 \times ODc) < OD \leq (4 \times ODc)$ = Strains with moderate biofilm forming ability

$(4 \times ODc) < OD$ = Strains with strong biofilm forming ability

Detection of biofilm associated genes

Primers for targeting biofilm associated virulence genes were used as per the literature (Table 3). The reactions were carried out in 25 µl volume in a thermocycler with conditions as: 95°C 5 min; 95°C 30 s, 55°C 30 s, 72°C 1 min, total 30 cycles; 72°C 5 min, holding at 4°C.

Table 3
Primers related to biofilm associated genes

Description	Literature	Gene	Primers (5'-3')	Product size (bp)
P fimbriae	(Wang et al. 2016)	*pap*C	F- TGATATCACGCAGTCAGTAGC R- CCGGCCATATTCACATAAC	482
Adhesive subunit of Type1 fimbriae		*fim*H	F- TGCAGAACGGATAAGCCGTGG R- GCAGTCACCTGCCCTCCGGTA	508
AI-2 signaling molecule		*lux*S	F- ATGCCGTTGTTAGATAGC R- CTAGATGTGCAGTTCCTGC	516
Curli gene	(Maurer et al. 1998)	*crl*	F- TTTCGATTGTCTGGCTGTATG R- CTTCAGATTCAGCGTCGTC	250
Curli subunit gene		*csg*A	F- ACTCTGACTTGACTATTACC R- AGATGCAGTCTGGTCAAC	200

Antimicrobial susceptibility testing

The isolates characterized as APEC were subjected to antimicrobial susceptibility testing (AST) using AST-GN 65 cards on Vitek 2 Compact. Interpretive breakpoints for susceptible, intermediate, and resistant were consistent with Clinical and Laboratory Standards Institute (CLSI) and Natural Resistance 2018 and were provided by the manufacturer. AST GN-65 contained 18 antibiotics along with ESBL detecting antibiotics for AST purpose. The ATCC 25922 was used as quality control. The testing was done as per manufacturers' recommendations and for the current study, APEC isolates with intermediate susceptibility were defined as not resistant and isolates were considered MDR if they were resistant to three or more antimicrobial classes (Magiorakos et al. 2012).

Statistical analysis

The data related to the characterization of APEC, biofilm detection and biofilm associated genes detection and antimicrobial susceptibility testing was analyzed by chi-square test using SPSS software. A value of $p < 0.05$ was considered as significant.

Results

Isolation and identification of E. coli

According to clinical symptoms, pathological lesions, selective media results, and Gram staining, 63 isolates were tentatively considered as *E. coli* from 114 samples processed. Further confirmation using Vitek2 Compact System and PCR identified 62 of these 63 isolates as *E. coli* (Figs. 1 and 2). The remaining one isolate was confirmed as *Enterobacter cloacae* by Vitek 2 Compact. In total, the test results indicated that 62 *E. coli* isolates were isolated from the samples collected from colibacillosis affected farms. Of these 62 *E. coli* isolates, 22 isolates were from diseased birds' tissues (liver, lungs, heart), six from cloacal swabs, while 34 isolates were from environmental sources at these farms viz., PVC pipes, feeder and drinker swabs, water, feed, litter, and tank wall surfaces.

Characterization of avian pathogenic E. coli **(APEC)**

Pentaplex and individual gene PCR amplification results using a set of five virulence genes (*iroN, ompT, hlyF, iss, iutA*) characterized 47 (75.8%) *E. coli* isolates as APEC and 15 (24.2%) isolates as non-APEC. The representative APEC isolates with five virulence genes are shown in Fig. 3. The pentaplex PCR exhibited the presence of the five genes among 45 APEC isolates while four genes in two APEC isolates. None of the tested genes was found in 11 non-APEC isolates. The prevalence of *hlyF*, *ompT*, *iroN*, *iutA* was 100% among 47 APEC isolates and for *iss* it was 95.7% (Fig. 10). The prevalence of the five virulence genes was found to be significantly associated with APEC isolates using chi-square test.

Biofilm detection and quantification

For effective comparisons, only moderate to strong biofilm producers were taken as biofilm positive isolates and weak biofilm producers were combined with non-producers. A total of 30 (63.8%) APEC isolates were moderate to strong biofilm producers of which six were moderate biofilm producers and 24 were strong biofilm producers and 17 (36.2%) APEC isolates were weak to non-producers (Fig. 4). Similarly, six non-APEC isolates out of 15 produced moderate-strong biofilms and nine were non-biofilm producers. Strong biofilm producing APEC isolates were obtained from cloacal swabs, heart, liver, litter, feeder, and drinker swabs.

Detection of biofilm associated genes

PCR amplification results targeting five genes (*crl*, *csgA*, *fimH*, *luxS*, and *papC*) (Figs. 5, 6, 7, 8, and 9) revealed the prevalence of *fimH* gene was significantly higher among APEC isolates. The prevalence of *crl* and *fimH* each was 95.7% among APEC isolates, followed by *luxS* (93.6%), *csgA* (61.7%), and *papC* (8.5%) (Fig. 10). On analyzing the association using chi-square test, it was found that the five genes used for characterization of APEC were significantly associated with each other and with the presence of *fimH* gene. The presence of *crl* gene among APEC was significantly associated with *csgA*, *ompT, fimH*, and *luxS*. In addition to this, there was a significant association of *csgA* presence with *fimH* and *luxS*.

Antimicrobial susceptibility testing

A total of 47 APEC isolates were tested for AST using Vitek 2 Compact (Fig. 11) and results (Fig. 12) showed maximum resistance for commonly used antibiotics viz., 89.4% for enrofloxacin and tetracycline, followed by marbofloxacin (85.1%), sulphamethoxazole/trimethoprim (70.2%), ampicillin (66.0%) and piperacillin (61.7%). The resistance observed for other antimicrobials was 38.3% for chloramphenicol, 25.5% each for amoxicillin/clavulanic acid, cefalexin and cefpodoxime, 23.4% each for cefovecin, ceftiofur and imipenem, 17.0% for tobramycin and 12.8% each for amikacin, gentamicin, and nitrofurantoin. Polymixin B was found to be 100% sensitive. A total of 43 isolates out of 47 were resistant to at least four antimicrobials out of 18 antimicrobials applied, indicating 91.5% isolates as MDR (multiple drug resistant). A total of 22 multiple drug-resistant phenotypes were observed. The most common forms of antibiotic resistant were to tetracycline, enrofloxacin, marbofloxacin, sulphamethoxazole/trimethoprim, ampicillin, and piperacillin (R-type: TEMStAP).

Eight APEC (17%) isolates were ESBL positive and the prevalence of ESBL positive isolates was significantly higher in the isolates resistant to penicillin derivatives viz., amoxicillin/clavulanic acid, piperacillin, ampicillin, and 3rd generation cephalosporins (cefovecin, ceftiofur, cefalexin and cefpodoxime). Significantly higher association ($p < 0.01$) of ESBL was also found with imipenem, gentamicin, and nitrofurantoin resistance.

A significantly higher antimicrobial resistance was found among the APEC isolates which formed moderate to strong biofilms compared to weak to non- producer isolates. The maximum resistance among biofilm producers was for gentamicin (100%) followed by amoxicillin/clavulanic acid, cefalexin, and cefpodoxime (91.7%). Similarly, 91.4% resistance was found for cefovecin, ceftiofur, and imipenem, among moderate to strong biofilm producers. Out of the total 30 moderate-strong biofilm producers, 26 (86.7%) were MDR, out of which 8 (30.8%) were resistant to more than nine antimicrobials. Out of 17 non-producers, 16 (94.1%) were MDR.

On analyzing antimicrobial resistance with the presence of the genes, it was found that *csgA* was significantly higher among tetracycline and sulphamethoxazole/trimethoprim resistant isolates ($p < 0.05$). The prevalence of *iss* genes was found significantly higher among isolates that were resistant to ampicillin and sulphamethoxazole/trimethoprim.

Discussion

In this study, we isolated 62 (54.38%) *E. coli* isolates from 114 different samples, which were confirmed by selective isolation on MLA and EMB, conventional PCR and Vitek2. Using a set of five virulence genes as described above, 75.8% isolates were characterized as APEC. The maximum APEC detection was from tissue lesions (100%) followed by environmental samples from the poultry house (73.5%) and cloacal swabs of affected birds (60%). The lower isolation of APEC from cloacal swabs of birds might be due to the use of antimicrobials in the feed and water of these farms (Hasan et al. 2011). Awawdeh (2018), reported that all the *E. coli* isolates from tissue lesions of affected birds (100%) were APEC, however, reporting higher (70%) APEC isolate from the feces of affected birds compared to this study (60%). The

APEC isolates obtained from the environment of the diseased flocks (73.5%) are higher than that obtained by Hussein et al. 2013 (approx. 30%) which might be due to the fact that the authors have limited the environment sampling only to litter and drinker swabs.

The prevalence of the five virulence genes was found to be significantly higher among APEC isolates. The maximum prevalence (100% each) was observed in the case of four genes viz., *hlyF, ompT, iroN, iutA*, and 95.7 % for *iss* gene. The *hlyF* gene is involved in the production of outer membrane vesicles which act as a channel to deliver virulence factors into the host (Murase et al. 2016), the *ompT* gene encodes the episomal outer membrane protease that cleaves colicins, *iroN* and *iutA* encodes for an iron acquisition system for bacteria and *the iss* gene encodes for a protein which inhibits the deposition of membrane attack complex (MAC) to provide serum resistance (Dziva and Stevens 2008). Among non-APEC isolates, the prevalence of *hlyF* and *ompT* was 26.7% each and *iroN* was 6.7%. The *iutA* and *iss* were not amplified from any of non-APEC isolates, suggesting their highly significant association with APEC isolates. Geographical variations in the prevalence of these genes have been reported. For example, prevalence of *hlyF* ranged from 24%-100%, *ompT* from 2.2%-100%, *iroN* from 84%-100%, *iutA* from 69–82% and *iss* 80–100% among APEC isolates (Johnson et al. 2008; De Oliveira et al. 2015; Awawdeh 2018; Subedi et al. 2018; Kazibwe et al. 2020). This study along with others suggests that the five genes used in the study are significantly associated to APEC isolates compared to non-APEC isolates (Johnson et al., 2008; Awawdeh 2018; Kazibwe et al. 2020). The high prevalence of these five genes which encode for various virulence traits suggests the pathogenic nature of APEC and these virulence genes (VGs) may be involved at different stages of infection in poultry birds. The frequency of APEC related VGs among *E. coli* strains varied and the virulence gene profile may be influenced by several factors such as geographical location, season, bird immune status, variation in sampling sites, different husbandry, and vaccination protocols (Johnson et al. 2008; Wang et al. 2016).

APEC biofilms in poultry settings have been reported as responsible for failure of disinfectants, failure of antimicrobials, and persistent *E. coli* infection of birds at the farm (Maharjan et al. 2016; Branco et al. 2016). We found in the present study that APEC (n = 30) and non-APEC (n = 6) isolates have the potential to form biofilms in vitro. Our biofilm results are close to the findings of Skyberg et al. (2007) and Wang et al. (2016) who observed 55.2% and 56.6% APEC isolates as moderate to strong biofilm producers. However, a lower numbers of biofilm producers were reported by Dou et al. (2015) and Oosterik et al. (2014), who found 36.2% and 44% APEC isolates as biofilm producers, respectively. This variation might be due to the different inoculating media used in these studies along with different incubation temperatures as adhesion of medium components such as amino acids or lipids to the abiotic surface may stimulate cell adherence and mucus-derived proteins may elicit bacterial cell agglutination as reported in previous studies using commensal and laboratory *E. coli* strains (Orndorff et al. 2004). However, our study along with previous investigations revealed that APEC isolates can form biofilm *in vitro* and thus can be one of the reasons of constant occurrence of colibacillosis in poultry farms.

Biofilm associated genes viz., *crl*, *csgA*, *fimH*, *luxS*, and *papC* were detected with high frequency among APEC and non-APEC in this study. The *csgA* was found to have a nearly similar distribution among APEC

and non-APEC isolates as reported previously (Maluta et al. 2014; Cunha et al. 2017). The significant association of *fimH* with VGs used for APEC characterization thus suggests that this gene can be used as a molecular marker for APEC characterization along with other VGs used for characterization (Schwartz et al. 2013).

The ubiquity of biofilm associated genes among APEC and non-APEC (Fig. 10) does not mean that these genes are not important for pathogenesis (Wang et al. 2016). All these biofilms associated genes have previously been reported among ExPEC strains of human and canine origin, which again suggests a possible linkage of APEC and ExPEC strains. Out of 30 moderate-strong biofilm producer APEC, two isolates were positive for the five genes, 17 isolates were positive for four genes, nine isolates were having three genes each, and two isolates were having two genes. Wang et al. (2016) also reported a high prevalence of biofilm associated genes among biofilm producing APEC isolates. However, it cannot be concluded that these biofilm associated genes are responsible for biofilm formation among these isolates as 14 APEC isolates, despite having more than three such genes, were either not able to produce any biofilm or only produce weak biofilms. This finding thus suggests that the mere presence of biofilm associated genes might not be attributed to biofilm formation, rather it is the level of expression of biofilm related genes, which might be temporally or environmentally regulated which should be assessed (Cunha et al. 2017; Rodrigues et al. 2018). Thus, quantification of the expression of biofilm related genes by real-time PCR or expression of curli and cellulose by using phenotypic methods is needed.

A total of 47 APEC isolates were tested for antimicrobial susceptibility to 18 antibiotics of GN AST 65 cards.). A total of 43 isolates (91.5%) were resistant to at least four antimicrobials. The higher resistance to antimicrobials is suggestive of rampant use of antibiotics in the commercial broiler industry as also concluded in the survey by Grakh et al. (2020A total of 22 MDR phenotypes and the most common form of antibiotic resistance were to tetracycline, enrofloxacin, marbofloxacin, sulphamethoxazole/trimethoprim, ampicillin, and piperacillin (R type: TEMStAP). In the present study, the significantly higher association of ESBL with imipenem, gentamicin, and nitrofurantoin resistant isolates is interesting as these are not beta-lactam antibiotics. Such an association of ESBL with the above said antibiotics is also previously reported in human medicine (Rawat and Nair 2010; Al-Zarouni et al. 2012). The plasmids carrying ESBL genes also carry genes for resistance to other antimicrobial agents such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol and thus confer co-resistance to these antibiotics indicating that a very broad antibiotic resistance extending to multiple antibiotic classes has become a frequent characteristic of ESBL producing *E. coli* isolates (Rawat and Nair 2010). Leverstein-Van Hall et al. (2011), revealed that ExPEC strains isolated from human patients shared the same ESBL genes and plasmids as carried by *E. coli* isolates cultured from retail chicken meat and colibacillosis affected birds, indicating that they can be easily transferrable among *E. coli* strains including human strains. Further high resistance against antibiotics was there in strong – moderate biofilm producing isolates as compared with weak or non-biofilm producing isolates. For instance, the six isolates which were resistant to gentamicin were strong biofilm producers. Previous investigations have suggested that biofilms confer resistance to bacteria by providing a platform for the exchange of resistance genes/plasmids (Romling and Balsalobre 2012; Wang et al. 2016).

Using chi-square test, a significant association of antimicrobial resistance was found with two virulence genes viz., *iss* and *csgA*. Significantly higher prevalence of *csgA* gene was found among tetracycline resistant and sulphamethoxazole/trimethoprim resistant isolates. And significantly higher prevalence of *iss* gene was found among isolates that were resistant to ampicillin and sulphamethoxazole/trimethoprim. Similar association of biofilm related genes with antimicrobial resistance was previously reported (Awawdeh 2018), and needs further studies to derive any conclusions. To the best of our knowledge, this is the first elaborative study on APEC characterization and biofilm formation in India. The constant occurrence of avian colibacillosis caused by APEC is a challenge to the poultry industry and leads to economic losses to stakeholders. The biofilm formation ability of APEC might be responsible for the constant occurrence of APEC and avian colibacillosis at these farm and antibiotic resistance of these isolates. Our study thus emphasizes the endemicity of biofilm producing and multiple drug resistant APEC infection in broiler chickens and their environment in Haryana. Further association and characterization of APEC and their comparison with ExPEC of human origin, might reveal their zoonotic potential, which may help to design prevention and control measures at poultry farms.

Declarations

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Conflicts of Interests/competing interests

The authors declare that they have no conflicts of interest regarding the publication of this paper. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who met the criteria for authorship but are not listed.

Availability of data and material

The data used to support the findings of this study are included within the article. Rest of the datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability Not Applicable

Author's Contribution

Dinesh Mittal and Anand Prakash designed the study along with Naresh Jindal. Kushal Grakh collected samples and performed the research work and prepared the initial draft for the manuscript. Dinesh Mittal and Anand Prakash analyzed and interpreted the data along with Kushal Grakh. All the authors prepared and reviewed the final draft.

Ethics Approval

No approval of research ethics committees was required to accomplish the goals of this study because sample collection was done after post-mortem of dead birds with consent from farm owners by veterinarians.

Consent to Participate Not Applicable

Consent for Publication Not Applicable

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Figures

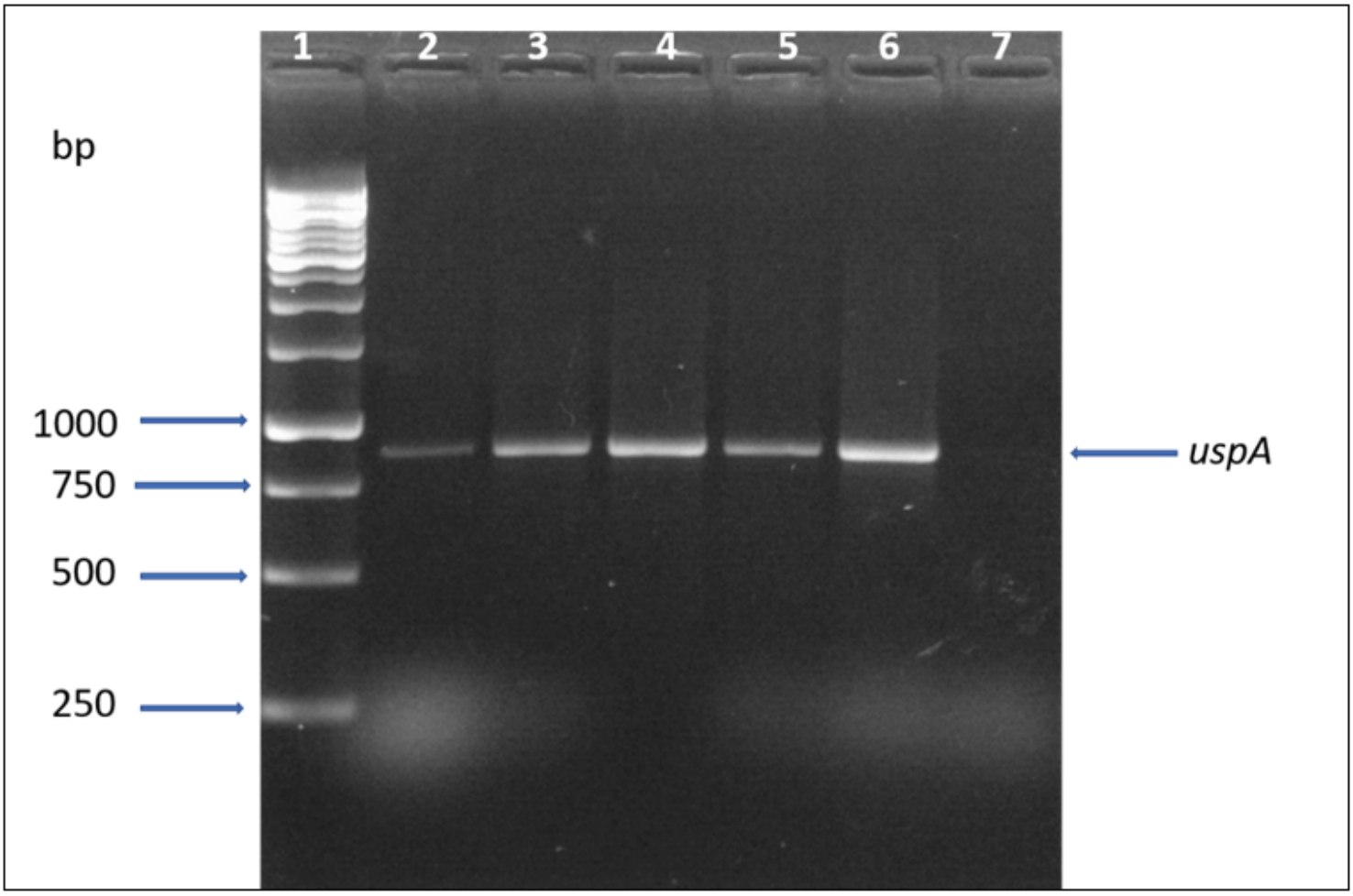

Figure 1

Agarose gel electrophoresis of the amplicon generated from uspA gene using PCR. Lane 1, Ladder; Lanes 2-5, uspA positive isolates; Lane 6, positive control ATCC 25922; Lane 7, negative control.

bioMerieux Customer:
System #:

Laboratory Report

Printed Aug 22, 2018 09:01 IST

Report Version: 1 of 1

Isolate Group: 5R L1-1

Card Type: GN Testing Instrument: 00001899ECFA (16209)

Identification Information	**Card:** GN		**Lot Number:** 2410488403	**Expires:**	Mar 29, 2019 12:00 IST
	Completed: Aug 21, 2018 16:05 IST		**Status:** Final	**Analysis Time:**	5.00 hours
Selected Organism	98% Probability	**Escherichia coli**			
	Bionumber: 0405610454566610			**Confidence:**	Excellent identification

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	+
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	-	47	ODC	+	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Figure 2

E. coli confirmation using Vitek2 Compact.

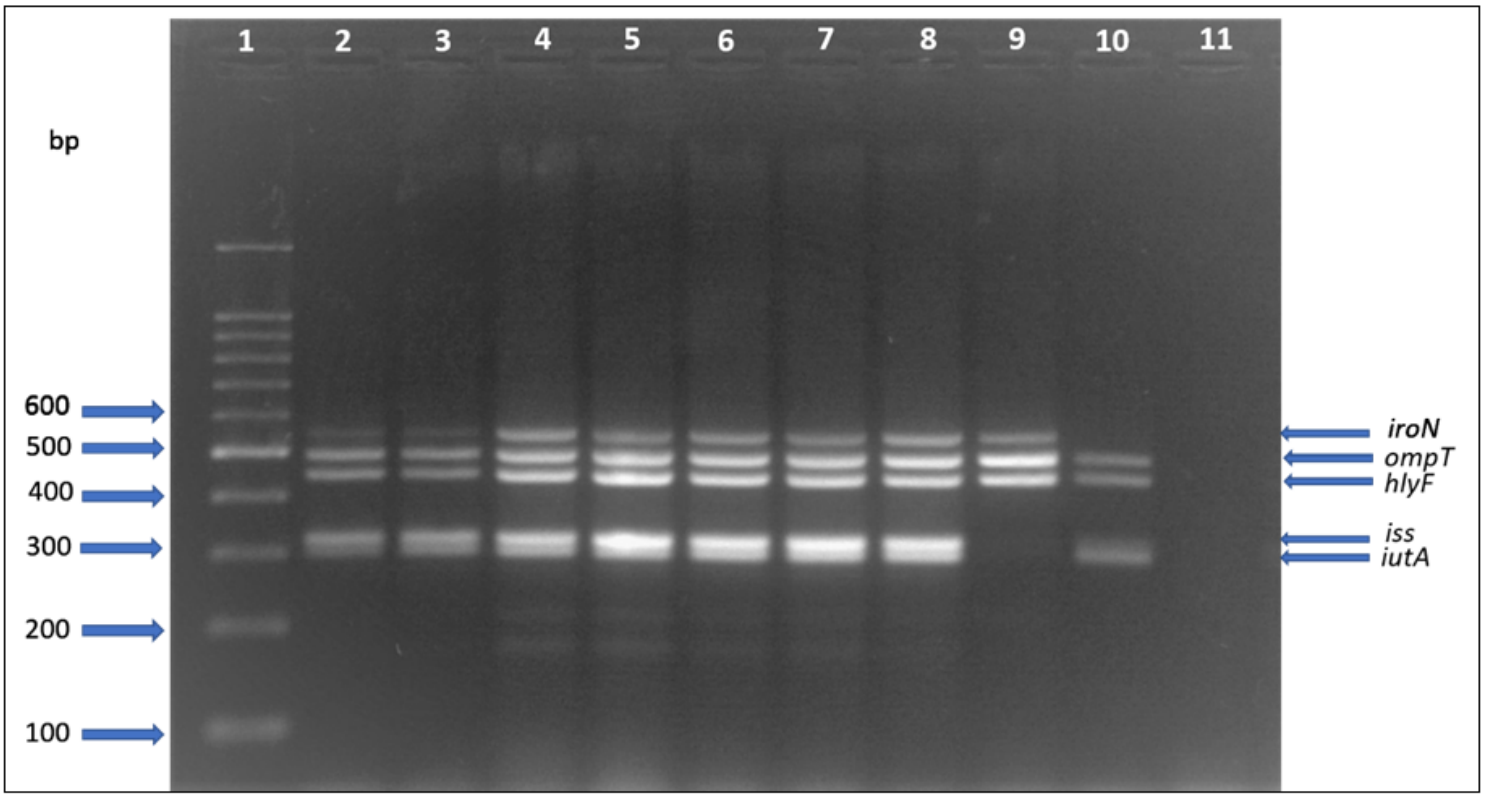

Figure 3

Agarose gel electrophoresis of amplicons generated in pentaplex PCR for APEC. Lane 1, 100 bp ladder; Lane 2, positive control (APEC O2); Lanes 3-7 and 10, APEC positive isolates; Lane 9, non-APEC isolate; Lane 11– negative control. Bands corresponding to iroN, ompT, hlyF, iss, and iutA are indicated.

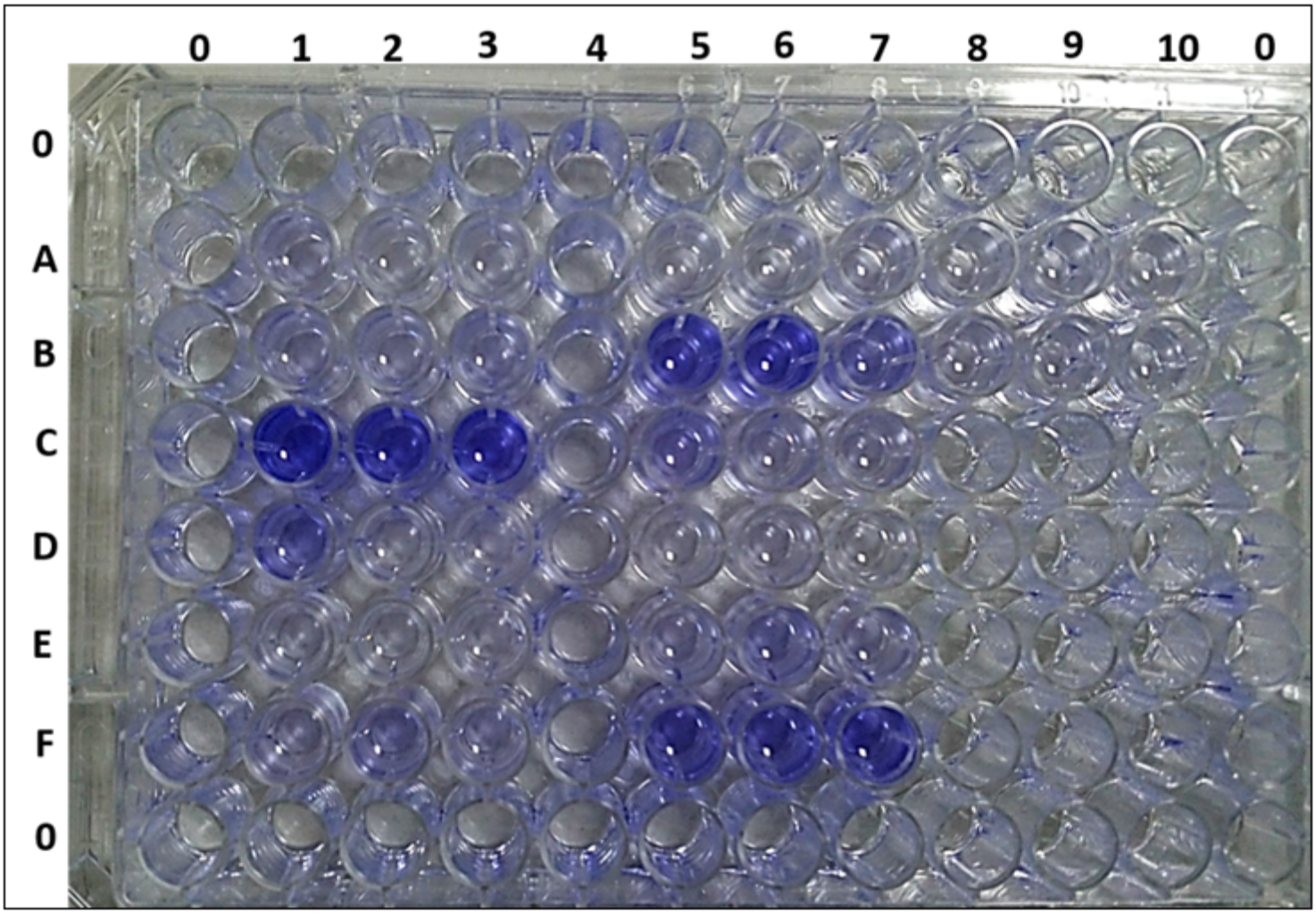

Figure 4

Biofilm in TSB media on a 96 microtiter plates. Row and column denoted by 0 are left blank. F5-F8: ATCC 25922 (positive control), A8-A10 and B8-B10: Negative control (uninoculated media), C1-C3 and B5-B7: Strong biofilm producer APEC isolate, D1-D3, F1-F3 and C5-C7, E5-E7: Intermediate biofilm producing E. coli isolates, A1-A3: Weak producer and E1-E3, A5-A7, D5-D7: No biofilms.

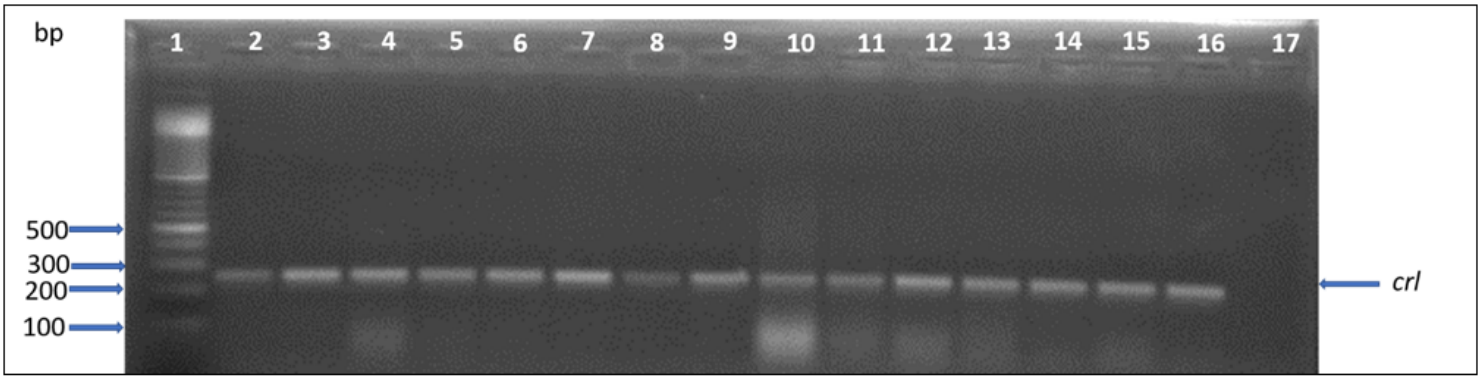

Figure 5

Agarose gel electrophoresis of the amplicon generated from the crl gene (250 bp) using PCR. Lane 1, 100 bp ladder; Lanes 2-15, crl positive isolate; Lane 16, positive control; Lane 17, negative control.

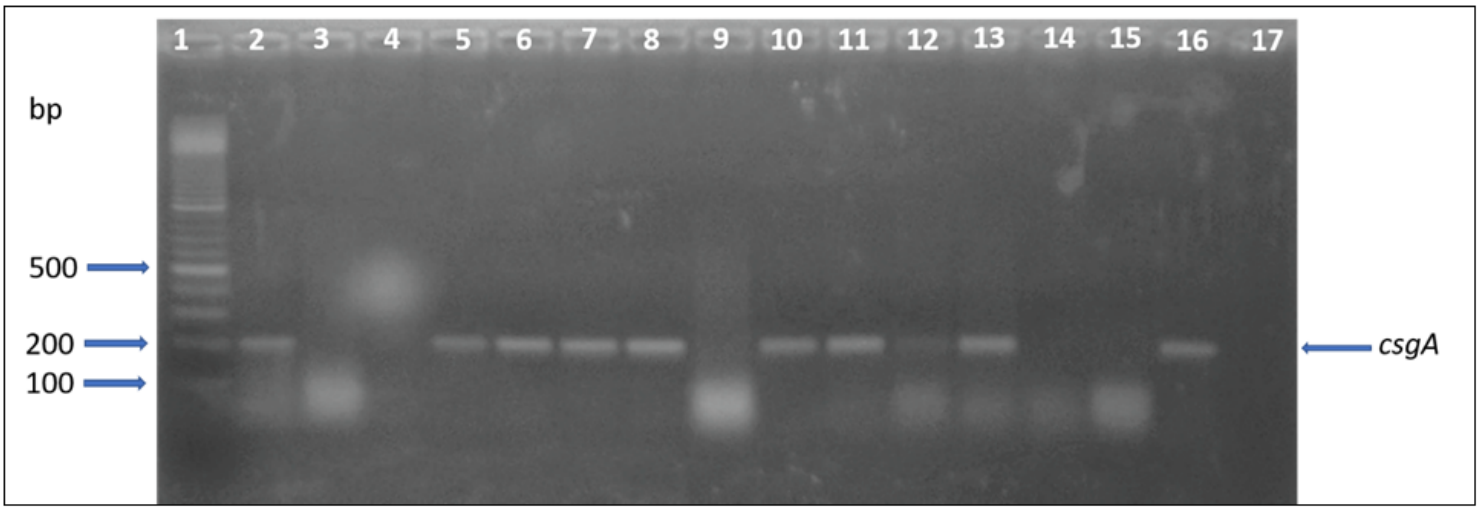

Figure 6

Agarose gel electrophoresis of the amplicon generated from csgA gene (200 bp) using PCR. Lane 1, 100 bp ladder; Lane 2, 5-8, 10,11 and 13 csgA positive isolates; Lane 3, 4, 9, 14, and 15, csgA negative isolates; Lane 16, positive control; Lane 17, negative control.

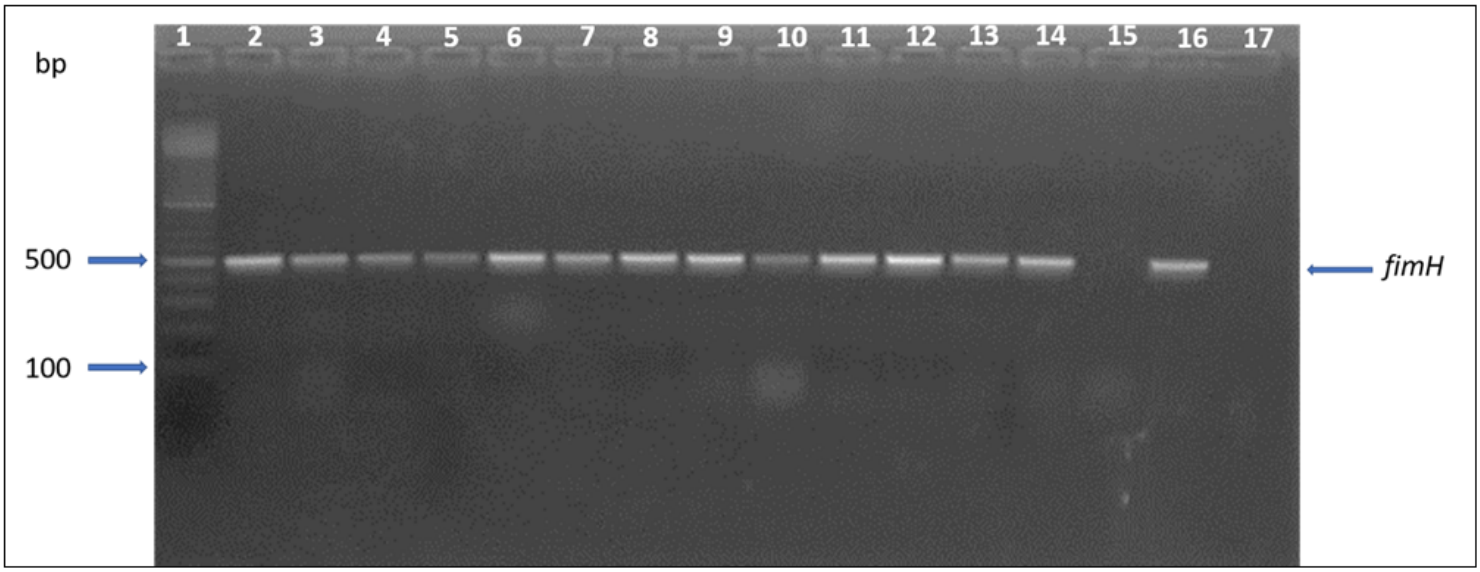

Figure 7

Agarose gel electrophoresis of an amplicon generated from the fimH gene (508 bp) using PCR. Lane 1, 100 bp Ladder; Lanes 2-14 fimH positive isolates; Lane 15, fimH negative isolate; Lane 16, positive control; Lane 17, negative control.

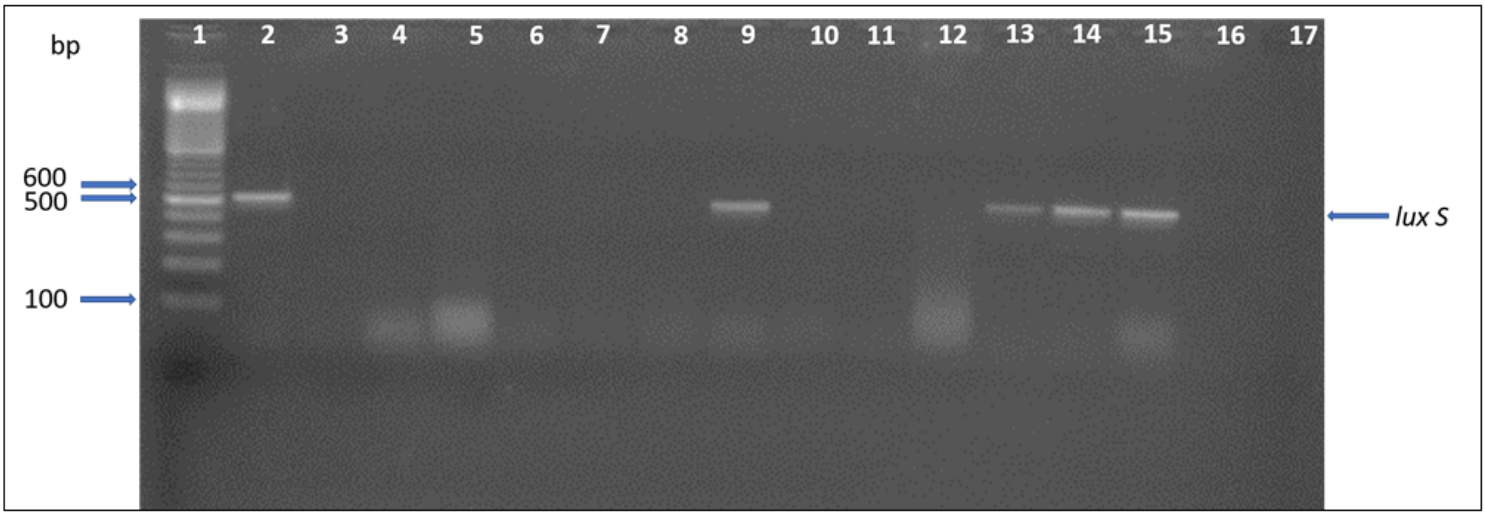

Figure 8

Agarose gel electrophoresis of an amplicon generated from LuxS gene (516 bp) using PCR. Lane 1, 100 bp ladder; Lanes 2, 9,13 and 14 luxS positive isolates; Lanes 3-8, 10-12, and 16 luxS negative isolates; Lane 15, positive control; Lane 17, negative control.

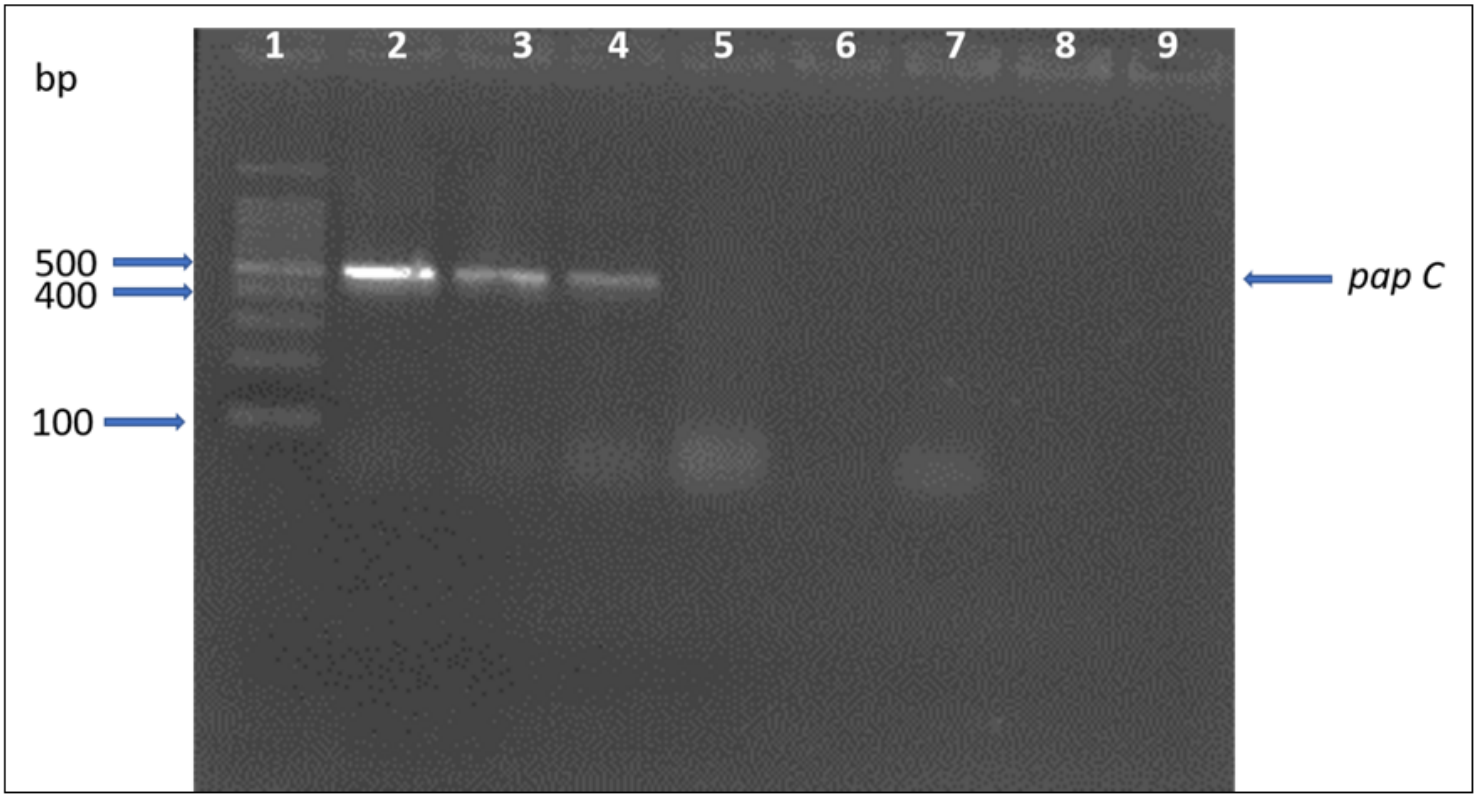

Figure 9

Agarose gel electrophoresis of an amplicon generated from the papC gene (482 bp) using PCR. Lane 1,100 bp ladder; Lane 3 and 4 papC positive isolates; Lane 5-8 papC negative isolates; Lane 1, positive control; Lane 9, negative control.

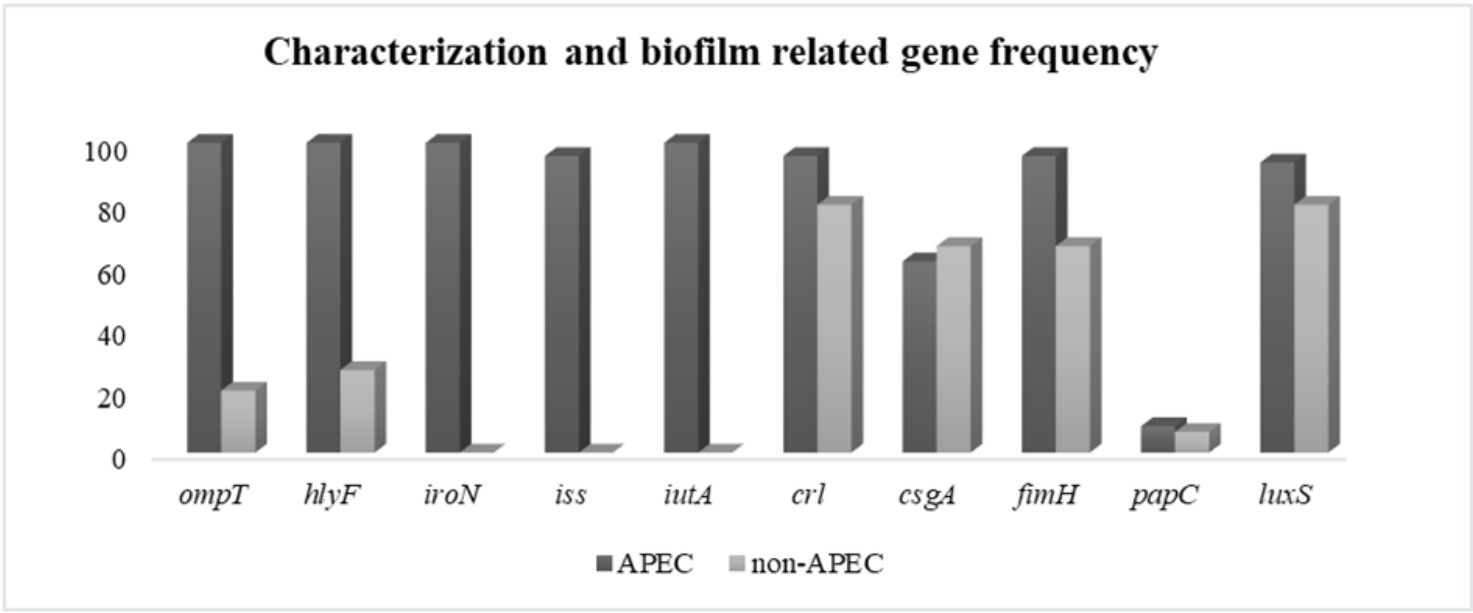

Figure 10

Distribution of genes used for characterization of APEC and genes related to biofilm in APEC and non-APEC isolates.

Isolate Group: 6R H-1

Card Type: GN Testing Instrument: 00001899ECFA (16209)
Card Type: AST-GN65 Testing Instrument: 00001899ECFA (16209)

Bionumber: 0405610450526610

Card is for veterinary use only

Susceptibility Information	Card:	AST-GN65	Lot Number:	5850716203	Expires:	Nov 12, 2019 12:00 IST
	Completed:	Aug 21, 2018 18:40 IST	Status:	Final	Analysis Time:	8.25 hours

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL	POS	+	Gentamicin	<= 1	S
Ampicillin	>= 32	R	Tobramycin	<= 1	S
Amoxicillin/Clavulanic Acid	[16]	*R	Enrofloxacin	<= 0.12	S
Piperacillin	>= 128	R	Marbofloxacin	<= 0.5	S
Cefalexin	>= 64	R	Tetracycline	<= 1	S
Cefpodoxime	>= 8	R	Nitrofurantoin	<= 16	S
Cefovecin	>= 8	R	Chloramphenicol	<= 2	S
Ceftiofur	>= 8	R	Polymyxin B	1	
Imipenem	[<= 1]	*R	Rifampicin		
Amikacin	<= 2	S	Trimethoprim/Sulfamethoxazole	<= 20	S

Figure 11

Antimicrobial sensitivity using AST-GN cards in Vitek 2 Compact.

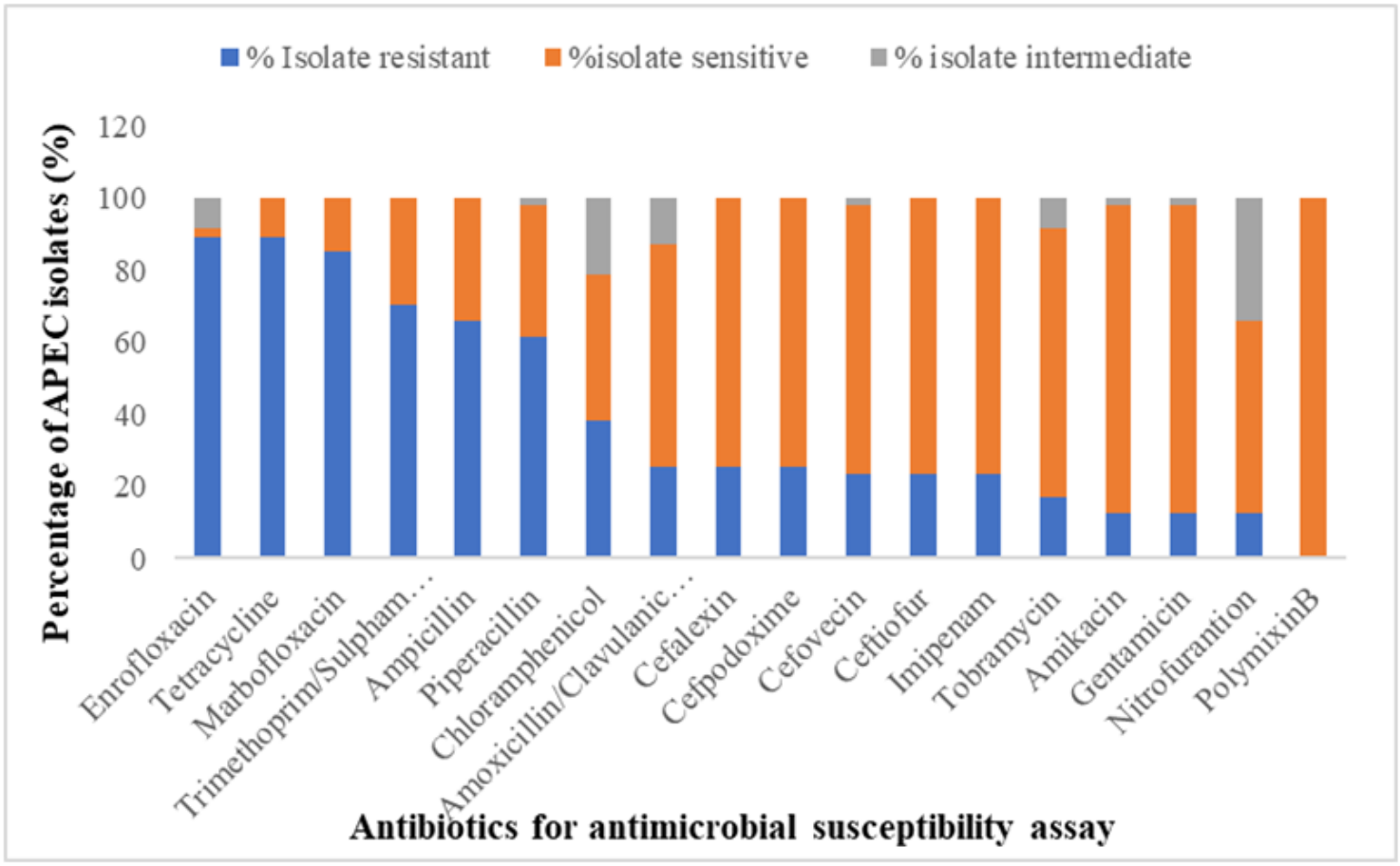

Figure 12

Drug sensitivity assay for APEC isolates. The columns denote the percentage of 47 APEC isolates that were resistant (blue), sensitive (orange), or intermediate (gray) to 18 common antibiotics.