Detection of virulence genes of APEC (avian pathogenic Escherichia coli) isolated from poultry in Noakhali, Bangladesh

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ABSTRACT: Avian colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC), is one of the major infectious diseases of poultry that bring about great economic loss for the Bangladesh poultry industry. The present study aimed to determine the virulence genes of avian pathogenic *Escherichia coli* (APEC) from cases of colibacillosis in poultry at the Noakhali district of Bangladesh. Currently, virulence-associated gene profiles of APEC isolates were investigated by polymerase chain reaction (PCR). A total of 24 (twenty-four) *Escherichia coli* isolates were collected and presumptively identified from 8 (eight) colibacillosis cases from 4 commercial broiler poultry farms (2 broilers per farm) in Noakhali, Bangladesh. The pathogenesis of *Escherichia coli* involves a wide range of different virulence genes. At this point, four virulence genes, iutA, hlyF, iroN, and iss were detected by PCR analysis. It has been observed that iutA, iss, hlyF, and iroN genes were found in 7(29.16%), 20(83.33%), 22(91.66%), and 24(100%) APEC isolates respectively. Furthermore, out of the twenty-four APEC isolates, six (25%) isolates had four virulence genes, fourteen (58.33%) isolates carried at least three virulence genes, three (12.5%) isolates carried two genes and one (4.16%) isolates had one virulence gene. Most importantly. six types of virulence gene profiles existed within the APEC isolates from which profile number 3 (hlyF, iroN, iss) having 13 (54.16%) isolates were predominant. The occurrence of APEC isolates of this region which is responsible for avian colibacillosis cases can be a matter of concern from the public health point of view. Future investigations will be able to utilize these virulence genes to identify APEC in Bangladesh helping in the diagnosis and prevention of colibacillosis in poultry.

KEYWORDS: APEC, Colibacillosis, Broiler, Virulence, PCR.

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Introduction

Poultry farming is one of the growing businesses in developing countries like Bangladesh with a significant impact on the regional economy by creating job opportunities (Islam *et al*., 2009). However, poultry diseases are the major constraints that hinder the productivity of the poultry industry in Bangladesh (Karim, 2003). Avian colibacillosis infection is highly prevalent in poultry of Bangladesh (Hasan *et al*., 2011). Colibacillosis is responsible for enormous economic losses in the poultry industry worldwide (Ewers *et al*., 2004; Ahmed and Shimamoto, *et al*., 2013).

Generally, *Escherichia coli* is a natural inhabitant of the gut in poultry and most other animals, but certain strains, such as avian pathogenic *E. coli* (APEC), spread into various internal organs and cause systemic fatal diseases (Oh *et al*., 2011). It was showed that APEC associated unusual disease in chicken such as swollen head syndrome, osteomyelitis (Stehling *et al*., 2003) while septicemia as well as polyserositis in broiler chicken to be associated with colibacillosis.

Basically, APEC strains pertain to cause diseases due to the expression of numerous virulence factors, such as adhesions, toxins, protectins, invasion, and iron acquisition mechanisms (Ghanbarpour *et al*., 2011). The virulence genes, such as iutA, hlyF, iss, and iroN are considered the most significantly associated with the pathogenesis of APEC (Johnson et al., 2008; Ahmed and Shimamoto, 2013). The iss gene found frequently in APEC strains that encodes the ISS protein which prevents the deposition of membrane attack the complex of complement system and increases serum survival (Rodriguez-Siek *et al*., 2005; Dziva and Stevens,2008; Johnson *et al*., 2008). The hlyF gene acts as avian hemolysin; iroN gene encodes IroN which acts as a siderophore receptor; and the iutA gene encodes ferric aerobactin receptor that involves in iron acquisition mechanism (Rodriguez-Siek *et al*., 2005).

Accurate diagnosis is the most important consideration in any poultry disease work and can reduce mortality problem. To the best of our knowledge, there is no report on the prevalence of these 4 genes (iutA, hlyF, iroN, and iss) in APEC isolates in Bangladesh. Therefore, the current study attempted to determine the virulence gene profiles of avian pathogenic *E. coli* (APEC) isolated from cases of colibacillosis in poultry farms in Noakhali, Bangladesh.

Materials and Methods

Collection of samples: Four commercial broiler poultry farms in the Noakhali region of Bangladesh were selected as sampling sites. All samples were collected aseptically from the liver, intestine, heart blood, and spleen from septicemia broilers (total eight broilers; two broilers from each farm) that died of colibacillosis. The samples were immediately transferred into buffer peptone water and were transported to the laboratory for bacteriological analysis at the department of Microbiology, Noakhali Science & Technology University.

Isolation and Identification of *E. coli*: Samples were plated on MacConkey agar (Oxoid, UK) and Eosin Methylene Blue (EMB) (Oxoid, UK) agar media and incubated at 37ºC for about 24 hours. *E. coli* were isolated and identified by cultural, microscopic, and biochemical tests described in Bergey's Manual of Determinative Bacteriology (Bergey and John, 1994). Biochemical tests were performed including TSI (triple sugar iron) tests, MIU (motility indole urease) test, methyl red, Voges-Proskauer, citrate, oxidase, and catalase tests.

DNA extraction: Total DNA was extracted using the boiling method outlined by B. Malorny (http://www.pcr.dk/DNA-purification.htm).

Gene-specific PCR for screening virulence gene:

PCR was carried out for four virulence genes of APEC: iutA, hlyF, iss, and iroN (Ewers *et al*., 2004; Ahmed and Shimamoto, 2013). PCR program was set as per protocol, in case of iutA, iroN and hlyF genes, 30 cycles (94°C, 30 s; 63°C, 30 s; 68°C, 2 min); for iss gene, 35 cycles (94°C, 30 s; 40°C, 30 s; 70°C, 2 min) ((Haghighi Khoshkhoo *et al*., 2019). Subsequently, the amplified PCR product was visualized by agarose gel electrophoresis (1.5% agarose gel). Primer sequences used for the detection of virulence gene of avian pathogenic *E. coli* (APEC) isolates and corresponding annealing temperature used for PCR, were summarized in Table 1.

Table-1. Primers used for PCR for amplification of APEC virulence genes

Primer Name	Sequence(5′→ 3′)	Target gene or region	Amplicon size (bp)	Annealing T (°C)	Reference
iroN-F	AATCCGGCAAAGAGACGAACCGCCT	*iroN*	553	63°C, 30 s	Johnson et al., 2008
iroN-R	GTTCGGGCAACCCCTGCTTTGACTTT				
hlyF-F	GGCCACAGTCGTTTAGGGTGCTTACC	*hlyF*	450	63°C, 30 s	Johnson et al., 2008
hlyF-R	GGCGGTTTAGGCATTCCGATACTCAG				
iss-F	CAGCAACCCGAACCACTTGATG	*iss*	323	40°C, 30 s	Johnson et al., 2008
iss-R	AGCATTGCCAGAGCGGCAGAA				
iutA-F	GGCTGGACATCATGGGAACTGG	*iutA*	302	63°C, 30 s	Johnson et al., 2008
iutA-R	CGTCGGGAACGGGTAGAATCG				

Results

The present study focused on the isolation of avian pathogenic *E. coli* (APEC) isolates from broilers of representative poultry farms in Noakhali, Bangladesh.

Isolation and Presumptive Identification of E. coli

Total of 24 bacterial isolates were isolated based on *E. coli* like colony characteristics on EMB and MacConkey agar. Colonies that resemble *E. coli* were selected for further Gram staining and biochemical tests. All isolates were Gram negative bacteria. Moreover, all these bacteria were triple sugar iron (TSI), indole, and methyl-red, catalase tests positive; whereas urease, citrate, H_2S, oxidase, and Voges-Proskauer tests negative; therefore, these bacteria were considered as *E. coli* presumptively (Supplementary Table 1).

The prevalence of virulence genes of avian pathogenic *E. coli* (APEC) isolates from broilers of four poultry farms was summarized in Table 2 and Table 3.

Table-2. Occurrence and Distribution of virulence genes of avian pathogenic E. coli (APEC) isolates from broilers of four different poultry farms in Noakhali, Bangladesh

Poultry Farm	Sample ID	iutA	iss	iroN	hlyF
A	AME1	-ve	-ve	+ve	-ve
	AIE2	-ve	+ve	+ve	+ve
	ABE3	-ve	+ve	+ve	+ve
	ARSE4	+ve	+ve	+ve	+ve
	AHBE5	+ve	+ve	+ve	+ve
	ASPE6	+ve	+ve	+ve	+ve
B	BME7	+ve	+ve	+ve	+ve
	BIE8	-ve	+ve	+ve	+ve
	BBE9	-ve	+ve	+ve	+ve
	BRSE10	-ve	-ve	+ve	+ve
	BHBE11	-ve	+ve	+ve	+ve
	BSPE12	-ve	+ve	+ve	+ve
C	CME13	+ve	-ve	+ve	+ve
	CIE14	-ve	+ve	+ve	+ve
	CBE15	+ve	+ve	+ve	+ve
	CRSE16	-ve	+ve	+ve	+ve
	CHBE17	-ve	+ve	+ve	-ve
	CSPE18	-ve	+ve	+ve	+ve
D	DME19	+ve	+ve	+ve	+ve
	DIE20	-ve	+ve	+ve	+ve
	DBE21	-ve	+ve	+ve	+ve
	DRSE22	-ve	-ve	+ve	+ve
	DHBE23	-ve	+ve	+ve	+ve
	DSPE24	-ve	+ve	+ve	+ve

Table-3. Frequency of virulence genes of avian pathogenic E. coli (AFEC) isolates from broilers in Noakhali, Bangladesh

Virulence genes	Number of positive strains (n = 24)
iutA	7(29.16%)
iss	20(83.33%)
iroN	24(100%)
hlyF	22(91.66%)

It was found that iron, hlyF, and iss genes were highly prevalent in APEC isolates, whereas the iutA gene had a low prevalent rate. According to the result, the percentage of iron, hlyF, iss and iutA genes were 100, 91.66, 83.33, and 29.16 respectively in APEC isolates (Figure 1).

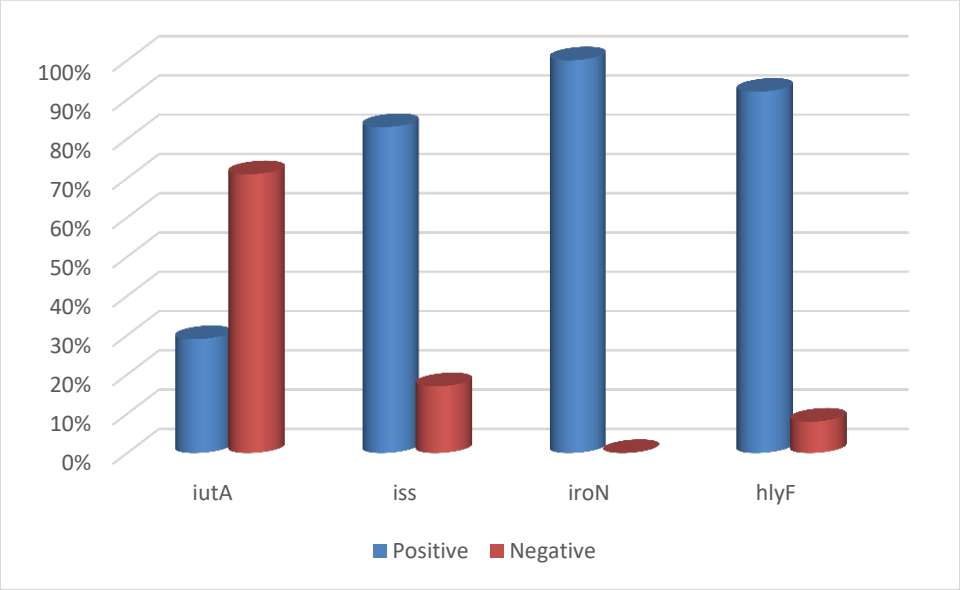

Figure-1. Percentage of virulence genes of avian pathogenic E. coli (APEC) isolates from broilers in Noakhali, Bangladesh.

Virulence-associated gene profiles

Based on the result, there were six types of virulence gene profiles (Table 4). Profile number 3 (hlyF, iroN, iss) having 13(54.16%) isolates was highly prevalent; in contrast, each of the profile number 2 (iutA, hlyF, iroN,), profile number 4 (iroN, iss) and profile number 6(iroN) having only one (4.16) isolates were less prevalent than other profile ones. Furthermore, profile number 1 (iutA, hlyF, iroN, iss), and profile number 3 (hlyF, iroN, iss) existed in all four poultry farms.

Table-4. Virulence-associated gene profiles of APEC isolates

Profile Number	Virulence Gene Profiles	Number of isolates	Number of isolates in each Farm				Percent
			A	B	C	D	
1	iutA, hlyF, iroN, iss	6	3	1	1	1	25
2	iutA, hlyF, iroN	1	-	-	1	-	4.16
3	hlyF, iroN, iss	13	2	4	3	4	54.16
4	iroN, iss	1	-	-	1	-	4.16
5	iroN, hlyF	2	-	1	-	1	8.33
6	iroN	1	1	-	-	-	4.16

Discussion

Avian pathogenic E. coli (APEC) affects broiler chickens of all period of ages when it carries virulence factors and come to intense decrease for the industry due to mortality, production losses, and condemnations (Wray *et al*., 2001). According to previous abroad studies, it is important to characterize APEC and identify virulence genes that can be used as molecular markers for the identification of APEC. However, there is inadequate data about APEC in broiler chickens in Bangladesh.

The present study investigated the prevalence of avian pathogenic E. coli (APEC) in poultry in the Noakhali district of Bangladesh. Samples were collected from eight broiler chickens with colibacillosis from four commercial broiler farms in Noakhali, Bangladesh. After presumptive identification by cultural, microscopic, and biochemical tests, DNA was extracted by the boiling method; the extracted DNA was screened by PCR for APEC-associated virulence genes.

This study identified a set of virulence genes: iroN, iss, iutA, and hlyF in the APEC isolates where the presence of iutA, iss, iroN and hlyF genes were 7(29.16%), 20(83.33%), 24(100%) and 22(91.66%) respectively (Table 3; Figure 1). In the United States, APEC strains (85.4%) were isolated from lesions of birds clinically diagnosed with colibacillosis having at least one of these 5 genes: iroN, iutA, iss, hlyF, and ompT (Johnson *et al*., 2008). In the current study, six different virulence gene

profiles were present in APEC isolates. In fact, profile number 1 (iutA, hlyF, iroN, iss) having 6(25%) isolates and profile number 3 (hlyF, iroN, iss) having 13 (54.16%) isolates were predominant. It was found that 6 (25%) isolates were positive for all four virulence genes; additionally, 14 (58.33%), 3 (12.5%), 1(4.16%) isolates had three virulence genes, two virulence genes, and one virulence gene respectively (Table 4). Previously, reports from several studies in agreement with these results. (Johnson *et al.*, 2008; Ahmed and Shimamoto, *et al*., 2013; Haghighi Khoshkhoo *et al*., 2019).

Although iutA, hlyF, iroN, and iss genes play an important role in the pathogenicity of APEC isolates, variances in the occurrence rates of these genes are reported. As the incidence of the disease is contingent on the host, pathogen, and environmental factors, any alterations in one of these factors can perhaps affect the occurrence of disease (Haghighi Khoshkhoo *et al*., 2019).

The iss gene present in APEC that is responsible for serum resistance. In this study, it was shown that iss gene was present in 83.33% of APEC isolated from avian colibacillosis. Several studies had presented that the prevalence of iss gene was reported in 72%-82% of APEC isolates (Jeffrey *et al*., 2002; Johnson *et al*., 2006; Johnson *et al*., 2008; Sampaio Baptista *et al*., 2010). The prevalence was 63% (Catana *et al*., 2008) in Hungary, 58.5% (Jin *et al*., 2008) in China. However, a lower prevalence for iss (38.5%.) has been observed from APEC isolated from poultry with Colibacillosis in Brazil (Delicato *et al*., 2003).

The iroN gene encodes IroN that acts as a siderophore receptor. The redundancy in iron-acquisition genes may play a role as an indicator of iron in the pathogenesis of avian colibacillosis. In our study, the prevalence of the iroN gene was 100% in APEC isolates. It was reported that the frequency of iroN in APEC isolates was 88.2% (Rodriguez-Siek *et al*., 2005); 67.2% (Johnson *et al*., 2006) and 85.5% (Johnson *et al*., 2008).

The hlyF gene encodes the putative avian hemolysin which is associated in the virulence of APEC. The prevalence of the hlyF gene in APEC was reported 78.2% and 81.7% (Johnson *et al*., 2008; Rodriguez-Siek *et al*., 2005) whereas in our current study, this prevalence was 91.66%.

The iutA gene encodes the aerobactin siderophore receptor that implicates the iron acquisition mechanism. The prevalence of the iutA gene was reported 78.0% (Ahmed and Shimamoto, *et al*., 2013), and 28.3% (Haghighi Khoshkhoo *et al*., 2019), in case of our study, it was 29.16%.

Therefore, it can be said that the prevalence of virulence genes may differ in various regions.

Conclusion

In conclusion, this study becomes noteworthy because it represents the prevalence of APEC and the presence of potential virulence genes of APEC in poultry at the Noakhali district of Bangladesh. Further, it is essential to conduct such a study in this region regularly to observe the status of APEC in poultry to ensure safety in poultry and public health.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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