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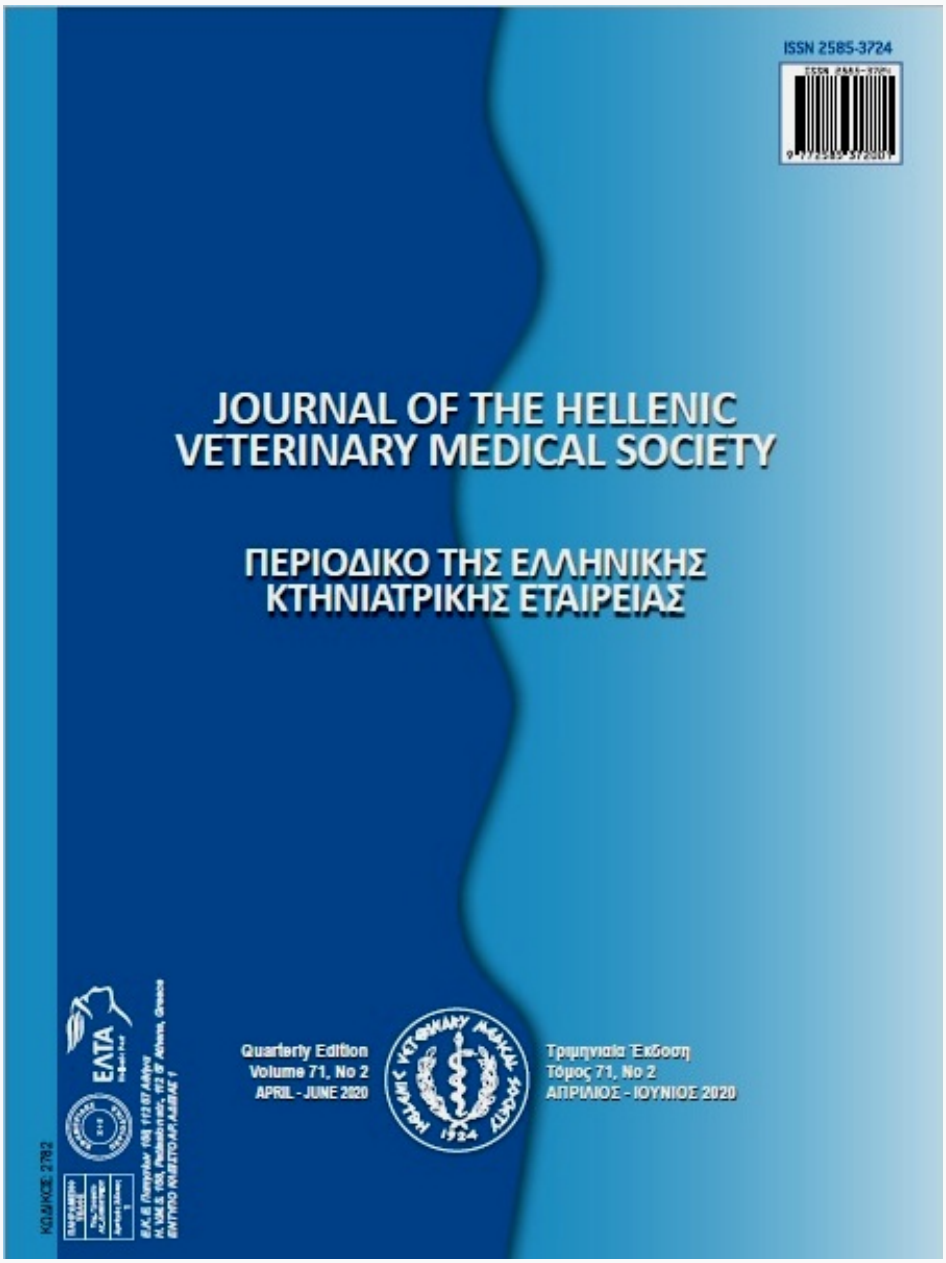

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Research article
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Molecular profile of avian pathogenic *Escherichia coli* (APEC) from poultry associated with colibacillosis in Algeria

M. Lounis[1], G. Zhao[2], Y. Li[2], Y. Gao[2], J. Wang[2], M. Oumouna[3], K. Oumouna[3]

[1] *Département des Sciences Agrovétérinaires, Faculté des Sciences de la Nature et de la Vie, Université Ziane Achour, Djelfa 17000, Algérie.*

[2] *Laboratory of Quality and Safety Risk Assessment for Animal Products, China Animal Health and Epidemiology Center, Qingdao, China.*

[3] *Faculté des Sciences, Université Yahia Fares, Médéa, Algérie.*

ABSTRACT: The objective of the present study was the detection of virulence-associated genes of *E. coli* isolated from chicken with colibacillosis. Seventeen (17) APEC isolates were examined by two panels of PCRs for the presence of 11 genes described for avian pathogenic (*hlyF, iroN, iss, ompT, iutA* and *fimC*) and diarrheagenic (*eae*, *stx*, *est*, *elt*, *ipaH* and *aggR*) *E. coli*. Results revealed that none of the APEC isolates harbored the genes *eae*, *stx*, *est*, *elt*, *ipaH* and *aggR*. In another hand, 88.2% of the isolates were positive for 3 or more of the virulence genes *hlyF, iroN, iss, ompT, iutA* and *fimC*. Also, 35.3% of the isolates harbored all the six genes. Genes *fimC* (88.2%), *iss* (82.3%) and *ompT* (76.5%) were the most prevalent while genes *hylF, iutA* and *iroN* which were present with the same frequency (52.9%) were mostly associated with highly pathogenic strains.

Key words: *E. coli*, APEC, virulence factors, avian colibacillosis, Algeria

Corresponding Author:
Lounis M., Département des Sciences Agrovétérinaires, Faculté des Sciences de la Nature et de la Vie, Université Ziane Achour, BP 3117, Route de Moudjbara, Djelfa 17000, Algérie.
E-mail address: lounisvet@gmail.com

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INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) are *E. coli* strains that can cause a localized or a systemic disease in birds of all ages named colibacillosis (Guabiraba and Schouler, 2015) one of the most important bacterial diseases in the poultry industry throughout the world (Kunert filho et al., 2015, Paixao et al., 2016). Avian colibacillosis is responsible for significant economic losses due to decreased egg production and hatching rates, morbidity, mortality, lowered production, carcass total or partial condemnation at processing and antibiotic treatment costs (Ewers et al*.,* 2004). Furthermore, the potential for zoonotic transmission must be considered, since poultry serves as the main host for APEC and the consumption of undercooked poultry may infect humans, which can serve as a reservoir of this pathotype (Kunert filho et al., 2015).

Long considered secondary pathogen, APEC has become in recent years accepted as a primary pathogen rather than a consequence of respiratory or immunosuppressive viral or mycoplasmal infections (Vandekerchove et al., 2004; Collingwood et al., 2014). This pathogen seems to be mainly restricted to a few O-serogroups where O1, O2, and O78 are the most common in epidemiological studies (Jeong et al., 2012; Kunert Filho et al., 2015)

Recently, multiple virulence factors were described in APEC including adhesins, toxins, iron uptake systems, invasins, autotransporters and resistance to the host serum (Ewers et al., 2004; Schouler et al., 2012).

However, no single common virulence factor has been identified in all APEC strains (Collingwood et al., 2014). Furthermore, some APEC isolates carry few, if any, of the most common APEC virulence factors and they are all rarely present in the same isolate (Collingwood et al., 2014; Guabiraba and Schouler, 2015).

Due to a lack of definitive consensus of classification APEC pathotype, multiple studies have attempted to define common associated virulence genes of APEC using essentially multiplex PCRs (Ewers et al., 2005; Johnson et al., 2008; Jeong et al., 2012; Schouler et al., 2012; Dissanayake et al., 2014). However, one of the most adopted studies is the work of Johnson et al (2008) based on the presence of five genes located on ColV plasmid and considered as potential markers for differentiation and identification of highly pathogenic APEC that has a strong potential of causing extra-intestinal diseases in birds (Johnson et al., 2008). These genes include the episomal outer membrane protease (*ompT*) that cleaves colicins, the outer membrane siderophore receptor gene (*iroN)* and the aerobactin gene *iutA (iron uptake transporter)* implicated in iron acquisition, the increased serum survival gene (*iss*) which has a role in the complement resistance and the new class of avian haemolysin gene (*hlyF)* implicated in the production of outer memebrane vesiscule, toxin releasing and contribute to iron uptake (Morales et al., 2004; Murase et al., 2016).

Other virulence factors like the type 1 fimbriae *fimC* are also highly associated with APEC (Ewers et al., 2004; Jeong et al., 2012). This gene is implicated in the adherence to host epithelial cells of the respiratory tract and colonization (Jeong et al., 2012).

On the other hand, it has been shown that APEC can harbor a number of virulence genes described for diarrheagenic *E. coli* like *eae*, *stx*, *elt*/*est*, *ipaH* and *aggR* (Hughes et al., 2009; Ramadan et al., 2016) suggesting its zoonotic potential and its possible risks to humans.

Little literature is available on molecular characterization of APEC strains isolated from Algeria. This study was carried out in order to provide more information on the virulence factors of APEC strains isolated from chicken with colibacillosis in Algeria.

MATERIALS AND METHODS

Bacteria

Seventeen (17) isolates were obtained from a diagnosis veterinary laboratory located in the department of Tizi Ouzou, Algeria. These isolates were previously isolated from birds (turkey, layer, breeders) with clinically symptoms of colibacillosis from different departments. The isolates were subcultured on Mac Conkey agar (Celmed Company, Algeria) at 37 °C for 18 to 24 h. One suspected colony was picked and subcultured on nutrient agar (Institut Pasteur Algeria) overnight at 37 °C. Isolates with typical characteristics were identified biochemically using API20E® system (Biomerieux, France). All *E. coli* isolates were stored at 4 °C until use. The biochemical identification was performed at the Laboratoire d'Hygiène Intercommunal, Draa El Mizan, Algeria.

DNA extraction

For the molecular detection of the genes, DNA of the *E. coli* isolates was extracted by boiling method

as described by Blanco et al (2004). *E. coli* isolates were, subcultured overnight at 37 °C in Trypticase Soy Broth (TSB) agar. A bacterial suspension was obtained by adding 200 μL of sterile water. Bacteria were boiled for 10 min to release the DNA and centrifuged at 10,000 rpm/5 min. The supernatant containing DNA was poured into a new microtube and stored at -20 °C until use for PCR analysis.

Polymerase chain reaction (PCR)

All *E. coli* isolates were analyzed by PCR for the presence of the virulence-associated genes. The detection of *hlyF*, *iroN*, *iss*, *ompT*, *iutA* and *fimC* was analyzed by simplex PCR as previously described (Jeong et al., 2012) (Table 1). The PCR reaction was carried out in 25 μL volumes using 200 ng of DNA, 12.5 μL of GO Taq® Green Mix (Promega), 10.5 μL nuclease-free water (Sigma-Aldrich) and 0.5 μL of each primer (10 μM). The cycling conditions consisted of a 5 min activation step at 95 °C followed by 35 cycles of 95 °C for 30 s, annealing temperatures (Table 1) for 30 s, an elongation step at 72 °C for 1 min followed by the final extension step at 72 °C for 10 min.

The prevalence of genes *eae*, *stx*, *est*, *elt*, *ipaH* and *aggR* were determined by multiplex PCR as described by Toma et al (2003) (Table 1). The amplification was performed in 25 μL volumes with 200 ng of DNA, 12.5 μL of GO Taq® Green Mix (Promega), 10.5 μL nuclease-free water (Sigma-Aldrich) and 1 μL of mixed primer (10 μM).

The PCR program consisted of a 5 min activation step at 95 °C, followed by 35 cycles of 95 °C for 30 s, annealing at 56 °C for 30 s, an elongation step at 72 °C for 1 min and a final extension step at 72 °C for 10 min.

The amplified products were separated by 1% ethidium bromide-stained agarose gel electrophoresis along with a 100-bp ladder (BIOWEST, Hong Kong, China) and visualized under UV light. An *E. coli* isolate was considered positive for the gene of interest if it produced an amplicon of the expected size (Table 1).

Statistical analysis

Statistical analysis was performed using Fisher's exact test. Data were considered as significant when the p value was ≤ 0.05.

Table 1. Primer sequences and annealing temperature for the targeted virulence genes

Gene	Primers sequences (3'-5')	Annealing (°C)	References
hlyF	For GGCGATTTAGGCATTCCGATACTC	59	Jeong et al., 2012
	Rev ACGGGGATCGCTAGTTAAGGAG		
iroN	For AAAGTCAAAGCAGGGGTTGCCCG	61	Jeong et al., 2012
	Rev GACGCCGACATTAAGACGCAG		
iss	For AGCAACCCGAACCACTTGATG	57	Jeong et al., 2012
	Rev TAATAAGCATTGCCAGAGCGG		
ompT	For ATCTAGCCGAAGAAGGAGGC	57	Jeong et al., 2012
	Rev CCCGGGTCATAGTGTTCATC		
fimC	For GGAAATAACATTCTGCTTGC	51	Jeong et al., 2012
	Rev TTTGTTGCATCAAGAATACG		
iutA	For GGCTGGACATCATGGGAACTGG	61	Johnson et al., 2008
	Rev CGTCGGGAACGGGTAGAATCG		
eae	For CCCGAATTCGGCACAAGCATAAGC	56	Toma et al., 2003
	Rev CCCGGATCCGTCTCGCCAGTATTCG		
stx	For GAGCGAAATAATTTATATGTG	56	Toma et al., 2003
	Rev TGATGATGGCAATTCAGTAT		
est	For TTAATAGCACCCGGTACAAGCAGG	56	Toma et al., 2003
	Rev CCTGATCCTCAAAAGAGAAAATTAC		
elt	For TCTCTATGTGCATACGGAGC	56	Toma et al., 2003
	Rev CCATACTGATTGCCGCAAT		
IpaH	For GTTCCTTGACCGCCTTTCCGATACCGTC	56	Toma et al., 2003
	Rev GCCGCTCAGCCACCCTCTGAGAGTAC		
aggR	For GTATACACAAAAGAAGGAAGC	56	Toma et al., 2003
	Rev ACAGAATCGTCAGCATCAGC		

Table 2: Presence or absence of virulence genes and genotype of APEC isolates

Strains	*iss*	*hlyF*	*ompT*	*iroN*	*iutA*	*FimC*	Genotype
1	-	-	-	-	-	+	*fimC*
2	+	+	+	+	-	+	*iss, hylF, ompT, iroN, fimC*
3	-	+	+	-	+	+	*hylF, ompT, iutA, fimC*
4	+	+	+	-	-	+	*iss, hylF, ompT, fimC*
5	+	-	+	+	+	+	*iss, ompT, iroN, iutA, fimC*
6	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
7	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
8	+	-	-	-	-	-	*iss*
9	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
10	+	-	+	-	+	-	*iss, ompT, iutA*
11	+	-	-	+	-	+	*iss, iroN, fimC*
12	+	+	-	+	-	+	*iss, hylF, iroN, fimC*
13	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
14	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
15	+	-	+	+	+	+	*iss, ompT, iroN, iutA, fimC*
16	-	+	+	-	+	+	*hylF, ompT, iutA, fimC*
17	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
Total	14	9	13	9	9	15	
Frequency (%)	82,4	52,9	76,5	52,9	52,9	88,2	

RESULTS

Prevalence of virulence-associated genes

In the present study, seventeen 17 APEC isolates were examined for the presence of 11 virulence-associated genes described as APEC specific (*hlyF, iroN, iss, ompT, iutA* and *fimC)* and diarrheagenic *E. coli* genes (*eae*, *stx*, *est*, *elt*, *ipaH* and *aggR*). The prevalence of each gene in APEC isolates is shown in Table 2. PCR analysis revealed that all the isolates had at least one of the APEC specific virulence factor while none of them harbored the diarrheagenic *E. coli* specific gene. Genes *fimC* (88.2%), *iss* (82.3%) and *ompT* (76.5%) are the most prevalent. The genes *hylF, iutA* and *iroN* were present with the same frequency (52.9%).

Association of the virulence genes:

Results show that 88.2% of the isolates had 3 or more virulence genes. Furthermore, 23.5%, 17.6% and 35.3% harbored 4, 5 and 6 genes (Table 3).

Table 3. Percentage of associations between the detected virulence-associated genes in APEC isolates

	iss	*ompT*	*hlyF*	*iroN*	*iutA*	*fimC*
iss	-					
ompT	70.6	-				
hlylF	52.9	58.8	-			
iroN	64.7	52.9	59.2	-		
iutA	58.8	64.7	47.1	47.1	-	
fimC	70.6	70.6	64.7	64.7	58.8	-

Regarding genes of the association of Johnson et al (2008), results show that 35.3% were positive for the five genes and all the isolates also harbored the gene *fimC* while 63.6% of the negative strains (strains without the combination of the five genes) harbored this gene.

Different combinations were also tested. Results show that the association *iss-ompT*, *iss-fimC* and *fimC-ompT* were the most prevalent (70.6%) while *iutA-hylF* and *iutA-iroN* were the less prevalent among APEC strains (Table 4).

Table 4. Number and frequency of virulence genes among APEC isolates

Number of genes	Number of positive strains	Percentage (%)
6	6	35,3
5	3	17,6
4	4	23,5
3	2	11,8
2	0	0,0
1	2	11,8

All the strains were farther classified according to the study of Johnson et al, (2008) on highly pathogenic APEC strains for those possessing the five genes. Results show that *hylF*, *ompT* and *iutA* were statistically associated with highly pathogenic strains (Table 5).

Table 5. Frequency of virulence genes among highly and moderate APEC isolates

	Highly pathogenic APEC (%)	Moderate pathogenic APEC (%)
iss	100	72,7
hlyF	100	36,4*
ompT	100	63,6
iroN	100	36,4*
iutA	100	45,5*
fimC	100	63,6

Results were compared using the Fisher's exact test *p < 0.05

DISCUSSION

E. coli is present in the normal microflora of the intestinal tract and in the environment of poultry; certain strains must possess specific virulence attributes to cause disease. APEC is a particular pathotype of *E. coli* that carries specific virulence genes which induce avian colibacillosis; an extraintestinal syndrome commonly encountered which has a major economic impact in the poultry industry through the world (Colingwood et al., 2014; Guabiraba and Schouler, 2015).

In this study, seventeen *E. coli* isolates were obtained from birds (turkey, layer, breeders) with confirmed cases of colibacillosis and were screened for 11 virulence genes.

Results show that *fimC,* which encodes a periplasmic chaperone that directs assembly of type 1 fimbriae was the most frequent gene detected in APEC isolates (88.2%) which is in accordance of the results of different studies in the world describing a prevalence exceeding 90% (Ewers et al., 2004; Won et al., 2009; Jeong et al., 2012; Dou et al., 2016; Paxiao et al., 2018). This gene however, has been also detected with high prevalence in non-pathogenic isolates (McPeak et al., 2005; Lounis et al., 2018; Paxiao et al., 2018) suggesting that *fimC* may not play an important role in the pathogenesis of avian colibacillosis.

The *iss* (increased serum survival) gene usually located on large ColV and ColBM plasmids encodes a protein that plays a role in serum resistance, protecting against the actions of complement, and contribute to increase in *E. coli* virulence in one day old chicks (Binns et al., 1979). Gene *iss* was one of the most prevalent genes (82.8%) in this study. Similar observations were also reported (McPeack et al., 2005; Hussein et al., 2013; Ahmed et al., 2013; de Oliveira et al., 2015; Dou et al., 2016 ; Lounis et al., 2018 ; Paxiao et al., 2018; Varga et al., 2018).

All these data suggests that *iss* may be critically important in the pathogenesis of avian colibacillosis. Several trials were done using this gene as a potential vaccine target in the protection of this infection (Lynne et al, 2006, Lynne et al, 2012).

The outer episomal membrane protein encoded by the gene *ompT*, was also detected with high prevalence (76.5%) in this study. *ompT* could play a role in adherence to eukaryotic cells and cleaves antimicrobial peptides, protamine, plasminogen and colicins and may be implicated in the pathogenesis of avian colibacillosis (Stumpe et al., 1998; Hejair et al, 2017). Results obtained in our study are consistent with other reports describing high prevalences of this gene (Johnson et al. 2008; Ahmed et al, 2013; De Carli et al., 2015; de Olivera et al., 2015; Sola-Gines et al. 2015; Dissanayake et al., 2016; Chalmers et al., 2017; Varga et al, 2018). Lower prevalence were also described by Li et al (2015) and Mbanga and Nyararai (2015). This gene has been also isolated among commensal-fecal strains with prevalences that can reach 60% (Jeong et al., 2012; Hussein et al., 2013; Mohsenifard et al., 2016; Lounis et al., 2018).

Genes *HlyF*, *iroN* and *iutA;* all implicated in iron uptake were detected with the same prevalence (52.2%). These prevalences are generally lower than those described in several publications (Johnson et al., 2008; Ahmed et al., 2013; Hussein et al., 2013; Li et al., 2015 ; Wang et al., 2015; Mohsenifard et al., 2016; Lounis et al, 2018).

However, these genes are more frequent in highly pathogenic isolates than the others isolates determined by the association of Johnson et al (2008). This suggests that these genes may play critical role in the avian colibacillosis pathogenesis.

It has been reported that highly pathogenic APEC lead to primary infections while less pathogenic strains only cause disease when the poultry are under severe stressful conditions such as other diseases and environmental stress factors. In this study, 88.2% of the APEC isolates harbored 3 or more of the virulence factors which are in accordance with the available literature (Ahmed et al., 2013). In another hand, only 35.3% of the isolates are positive for the combination of the five genes *iss-ompT-hylF-iutA-iroN.* Higher frequencies of this combination were reported

in APEC isolates through the world with prevalence from 57.6% to 91% (De Carli et al., 2015; De Olivera et al., 2015, Hussein et al., 2013; Lounis et al., 2018) while Li et al (2015) found that four isolates (4.6%) only among 87 APEC harbored these combination. Our results revealed that not all the APEC isolates are equally virulent.

Regarding the specific genes of diarrheagenic *E. coli*, our results shows that all the 17 APEC isolates were negative the genes *eae, stx, elt/est, ipaH* and *aggR*. Similar to the results of current study, none of the isolates from septicemic broilers and quails harbored *ipaH*, *stx1*, *stx2*, and *eaeA* genes (Ghanbarpour et al., 2010; Salehi and Ghanbarpour, 2010). In a previous study, Lounis et al (2018) reported that all the 92 APEC strains tested are negative for *eae* and *aggR* genes while 5.4%, 2.1% and 2.1% were positive for the genes *est/elt, stx* and *ipaH* respectively. These results suggest that APEC strains have a lower potential to cause diarrhea in human.

CONCLUSIONS

In conclusion and despite the potential biases related to the relatively small sample size of APEC isolates; this study could contribute to the molecular characterization of APEC in Algeria.

Results of the prevalence of the virulence associated genes of APEC in this study are generally lower to those reported in several countries.

Concerning the specific genes of diarrheagenic *E coli*, our results show that APEC isolates have a low potential in inducing diarrhea to humans

Other studies using a large sample size are needed which could provide more informations and definitive conclusions about the molecular profile of APEC.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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