Ann. For. Res. 66(1): 533-543, 2023
ISSN: 18448135, 20652445

ANNALS OF FOREST RESEARCH
www.e-afr.org

VIRULENCE- ASSOCIATED *E. COLI* GENES *ISS, FELA, CVAC, TSH, IUTA* AND *KPSII* IN AVIAN RESPIRATORY ISSUES ISOLATED PATHOGENIC *E. COLI* STRAINS

Majid Hameed Hasan
Department of Microbiology, College of Veterinary medicine. University of Thi-Qar, Shatra, Iraq, Corresponding author: Majid_8229652@utq.edu.iq

Abstract. The avian-pathogenic *E. coli* strains virulence methods have searched constantly which had been shown to be multifactorial and specific properties of virulent bacteria were associated with isolated samples from highly infected avian cases. On this observe a complete of 10 isolates of *E. coli* from chicken flocks IRAQ sources with respiratory problems instances had been tested by PCR test to diagnose the existence of genes liable of the serum-resistant (*iss*), fimbrial adhesion (*felA*), colicin manufacturing (*cvaC*), temperature-sensitive hemagglutinin (*Tsh*), aerobactin (*iutA*) ,and *K1* and *K5* antigens of capsul (*kpsII*). The *iss* gene had been discovered in 0% of strains, *felA* in 0%, *cvaC* in 30%, *tsh* in 80 %, *iutA* in 100 %, and *kpsII* in 0%.

Keywords: E. Coli, Iss, FelA, CvaC, Tsh, IutA, KpsII

Introduction

In farms of poultry , the most prevalent microorganisms inducing infections is *E. Coli*. The method applied to classify of the infectious *E. Coli* bacteria is depending on the obvious virulence is: enteropathogenic, enterotoxigenic,and enterohaemorrhagic and they are called virulent strains of *E. Coli* or avian pathogenic *E. Coli* (APECs). Colibacillosis induced by APEC in birds remains a major source of worry due to the financial losses it causes in poultry products of all kinds, where growth technique plays an fundamental role [1–3]. Avian colibacillosis or colisepticemia has different clinical symptoms and lesions depending on the age of the bird [4]. In broilers , an severe septicemia with big liver and spleen while in the form of subacute infection there are pericarditis, airsaculitis, arthritis, pneumonia and salpingitis [5]. The pathogenic nucleus and plasmid genes of avian *E. Coli* strains determine the virulence of strains [4,6]. Mostly each avian pathogenic E. Coli isolates has no less than 13 pathogenicity liable genes [7,8].

Through of the molecular studies of the genetic relatedness between the strains of APEC, the result of these scientific researches was the determinants of the virulence that depend on the frequency in APEC isolates that maybe individually or polygenically , depending on frequency in the APEC isolates [9]. Different virulent genes associations in APECs are existing and rarely samey [10,11]. It's very seldom that the genes of virulence exist all in the strain itself, confirming the strain of bacteria is a heterogeneous group. Applying of Polimerase Chain Reaction test to analyze APEC pathogenic genes , we can discover the genes as : *iss, cvi, iutA, hlyF, tsh, ompT, iucC , iroN,* [12–

14]. In this study we has searched of 6 virulence genes of APEC depending on the PCR test, the gene of bacterial fimbrial adhesion, gene of colicin manufacturing, gene of aerobactin existence, gene of serum resistance, gene of temperature-sensitive hemagglutinin, and *K1* and *K5* capsul antigens existence gene to characterize and differentiate several Iraq strains of the APECs. The 6 genes which were chosen, were identified in APECs as virulent genes previously [15,16].

MATERIALS & METHODS

Samples of Bacteria

Ten *Escherichia coli* strains from chickens flocks of different ages (days -35 wks of different regions of Baghdad province,Iraq) which were had respiratory symptoms and the anatomical lesions of colisepticemia. The collected samples were from different holding categories, broilers and layers. The cultivation of *E. Coli* strains were done on nutrient broth ,nutrient agar, MacConkey agar and Eosin Methylene Blue (EMB) Agar to be sure they are *Escherichia coli*. For the isolated *E. coli* virulence genes molecular identification, the enzymatic amplification reaction in the chain was performed. Strains of E. coli were cultured in broth of brain heart (BHI) for 24 hrs at 37 °C. Inoculation of the the culture into plates of MacConkey agar was done and incubated for 24 hrs at 37 ° C. Harvesting of the colonies were done and they had been stored at -20 ° C.

Virulence genes

E. Coli isolates had been tested by the PCR test to diagnose the existence of the 6 virulent genes , the fimbrial adhesion e F11 (*felA*), colicin manufacturing (*cvaC*), gene of aerobactin existence (*iutA*), gene of serum-resistant *(iss),* gene of temperature-sensitive hemagglutinin (*tsh*), and gene of *K1* and *K5* of capsul antigens existence gene (*kpsII*).

The PCR test

Extract of the deoxyribonucleic acid (DNA)

The DNA extraction had performed by using kit of the QIAamp Cador Mini (Qiagen, Dusseldorf, Germany) according to the manufacture instructions . The QIAamp Pathogen Mini Kit from various samples was used for the extract of nucleic acids such as bacterial DNA from a variety of samples . Under denaturing conditions at room temperature (15 - 25 ° C) using proteinase K and VXL buffer the samples were lysed . Both protein K and VXL buffer inactivated nucleases. The conditions of binding for DNA purification will be strengthened the moment that the buffer CBA is added. The transferring of Lysate to the column then was done and centrifugation of the nucleic acids had been adsorbed by membranes of silica and the contaminants will be removed . The membranes of silicate had been exposed to two procedures of washing to eliminate the pollutants remained and the restraints of enzyme. After adding of the buffer AVE, removing of nucleic acids was gotten followed by keeping of them in -80 C for a long-lasting time and in a

refrigerator temperature, if keeping for a short time. The protocol of PCR amplification was implemented as follows: 5 minutes at 94 C, pursued by 35 revolutions for half a minute at 94 C, three-quarters of a minute at 63 C, and one minute and three-quarters of a minute at 72 C. For 10 minutes there was a final elongation step was done under 72 C. Applied Biosystems ABI 2720 Thermal Cycler was used as thermocycler . The final volume that the reaction mixture was made up to is 50 µl din of: 2 DNA templates, 35.5 µl RNase-free water, 2 µl 10 mM dNTP, 2 µl polymerase of Taq platinum (5 U / 11 1) (Invitrogen®, Itapevi, São Paulo, Brazil), 5 µl the buffer of PCR (50 mM KCI, 10 mM Tris-HCI, pH 8.0), 1.5 µl MgCl2 (1.5 mM) and 1 µl before the primers and vice versa for all of the six genes checked (10 µmol). The primer sequence was that of [17]. An amplification reaction combination was done individually for each of the six virulence genes investigated. 1.5 percent agarose electrophoresis at 90 V and 1.5 A for 35 minutes was used to visualize amplicons. Table 1 lists the primers used in PCR, as well as the sequences of primers used before and after, to amplify six pathogenic genes segments and their predicted sizes followed by table 2 which lists results of the PCR test application pursued by the table 3 which shows frequencies of pathogenic genes results.

RESULTS AND DISCUSSION

High number of researches have emphasized that APEC has different virulent methods and the interaction of the several pathogenic elements resulted in the strains existence of *E. Coli*. Some specialists devoted their researches to this subject and what related to it, and despite the differences in the frequency , the main pathogenic elements which are; fimbrial adhesion , sensitive temperature hemagglutinin , increased serum sevival, aerobactin existence, colicin manufacturing, and the existence of certain antigens of capsul are important [1,10,18–25]. The determiners of genes that are accompanied by proteins situating in plasmids that exist in colicin producing samples are called Col factors [26]. Mainly, Colicin V is available in pathogenic E. coli implicated in the infections affecting humans and animals [27,28] and it suppresses the bacteria growing and disserve bacterial membrane formation

Table 1. The primers sequence & amplified fragments sizes used in polymerase chain reaction for the finding out of the APEC six virulence genes.

Primers names (pb)	Primers sequences (5° − 3°)	Products sizes (bp)
feIA	GGC AGT GGT GTC TTT TGG TG (F) GGC CCA GTA AAA GAT AAT TGA ACC (R)	270
cvaC	CAC ACA CAA ACG GGA GCT GTT CTT CCC GCA GCA TAG TTC CAT	680

Iss	GTG GCG AAA ACT AGT AAA ACA GC CGC CTC GGG GTG GAT AA	720
iutA	GGC TGG ACA TCA TGG GAA CTG G CGT CGG GAA CGG GTA GAA TCG	300
tsh	GGT GGT GCA CTG GAG TGG AGT CCA GCG TGA TAG TGC	620
KpsII	GCG CAT TTG CTG ATA CTG TTG CAT CCA GAC GAT AAG CAT GAG CA	272

bp= base pair, primer sequences [17,29,30]

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Yang, et al., [31] study the gene, *cvaC* was discovered in 30% of the tested strains . Some researchers found lower than this level result as registered in Spain,it was (22%) [32], in Brazilan isolated strains the gene has been discovered in 35% [10], while other researchers obtained higher percentage like [25,33] were reported 66.8%. and 99.1% respectively . The reports results variation could be due to the regional differences and samples sizes tested . As well 8 isolates (80%) accommodated the *tsh* gene which responsible of encoding of auto-transporter protein existed in APEC as Sarowska et al., [34] proved.

According to Hasani et al. [35] there was a propagation value of the gene *tsh* of the 71 studied virulence strains of APEC that was 49.3% which were registered in Iran and was highly lower than we registered. In Korea, Won et al., [36] was declared 55% value of the gene *tsh* of 118 virulent studied strains. The relative low prevalence of the *cvaC* gene which was reported to be connected with the *tsh* gene low prevalence value may be the reason behind the low prevalence value of *tsh*. It has been reported its role during of infections due to APEC, pathogenesis in acute infection , and its association to col V when both of them were available on the plasmid itself [37]. It participates in air sac lesions developing and is correlated with the high pathogenicity among the strains of APEC. The resistance of serum is depended on the capsule structural surfaces which is lipopolysaccharide, the outer membrane protein and any else protein membranes [20] and the two antigens K1 and K5 which the genes *kps* are codifying of them [8]. Some of the plasmids are able to transfer resistance of serum to the cells of the sensitive receptor . The *iss* genes were recognized in plasmids of pCol V-I-K94 and to the protein which is related to , the cytotoxic complex inhibition [38]. The gene of *iss* was not predominant in all of the ten APEC tested strains (0%). It's recognized to be related with the resistance of serum [39]. It's the more considerable connected gene with the strains of APEC [40]. The gene of *iss* had been found out in a high rate of extra-intestinal strains of infected birds (72.2%) when they were compared to the strains of the intestine (0%) and this confers insight for the significant of its virulence [41]. Dissanayake et al. [40] had been declared a higher positive rate for the gene of *iss* in the USA, than what we registered, which was 80.5% of USA APEC isolates. The results of other researchers were different from the result of our research about the *iss* percentage as example the rates (72.8%),

(77%) and (81.5%) which were declared by the researchers [25,33,42], respectively. The rate was (0%) positive for the *kpsII* gene of our analyzed samples . The genes of *kpsll* are liable about antigens coding K1 and K5 [43]. The K1 antigen is suspected being an significant pathogenic element of *E. Coli* while the K5 antigen exists in extra-intestinal infectious *E. Coli* strains [34]. The declared result of *kpsll* gene (18%) of Brazilian strains by Rocha et al. [30] was much higher than of we detected (0). The iutA gene is an important gene that liable with another genes about siderophores encode (aerobactin operon). The invasive *E. Coli* is responsible of producing the aerobactin. The aerobactin system gives the microbes the ability to live in media have no iron [44]. The system of aerobactin has an importance in lesions emergency, development and immutability in infected poultry with APEC [45]. The declared rates of *iutA* gene by other researchers [45,46] declared the rate 96% and the rate of 80% respectively were lower than what I detected (100%). *felA* is the operon that controls the (F11) P fimbriae serological variant [47]. In our study, the rate 0% was positive for the gene of *felA*. Very different rates of felA gene had been declared by the researchers in the world . For examples, [30,33,48], had declared a higher predominance rate of 78% in the United Kingdom, a low predominance rate 38.8% in Brazil and very low predominance rate 2% in Iran as listed in Tables (2 and 3).

Table 2. The positive and negative results of the detected strains for the existence of six pathogenic *Escherichia coli* genes.

Tested strains										
Detected Genes	1	2	3	4	5	6	7	8	9	10
1 *felA* 270 bp	-	-	-	-	-	-	-	-	-	-
2 cvaC 680 bp	-	-	-	+	-	+	-	-	+	-
3 *iss 720* bp	-	-	-	-	-	-	-	-	-	-
4 *iutA* 300 bp	+	+	+	+	+	+	+	+	+	+
5 *tsh* 620 bp	+	–	+	+	+	+	+	-	+	+
6 *KpsII* 272 bp	-	-	-	-	-	-	-	-	-	-

Table 3. The Virulence genes frequencies of of tested avian pathogenic *Escherichia coli strains*

feIA	*cyaC*	*iss*	*iutA*	*tsh*	*KpsII*
0%	*30%*	*0%*	*100%*	*80%*	*0%*

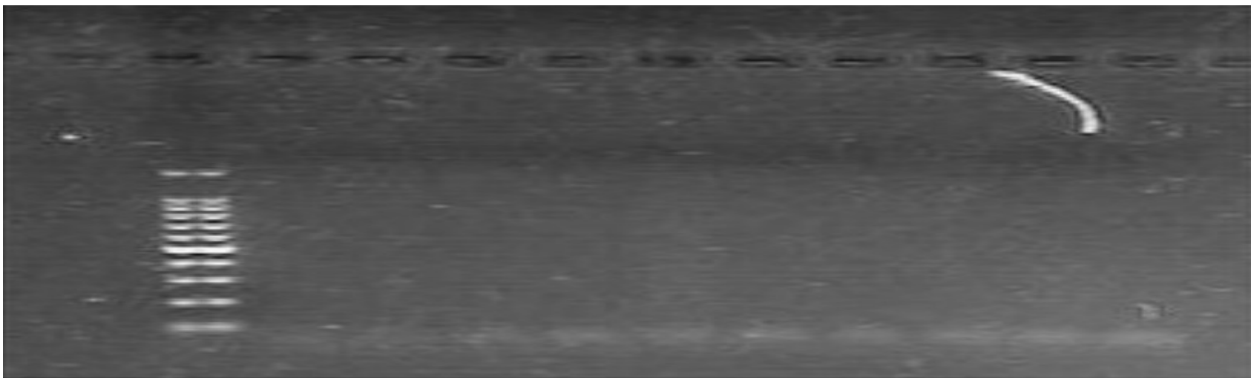

FIGURE 1. Exploration of ***felA*** gene in isolates (1-10). (+) isolates give line or stripe (270 basepair); lane M: 1Kb ladder of DNA ; lanes were (-) isolates.

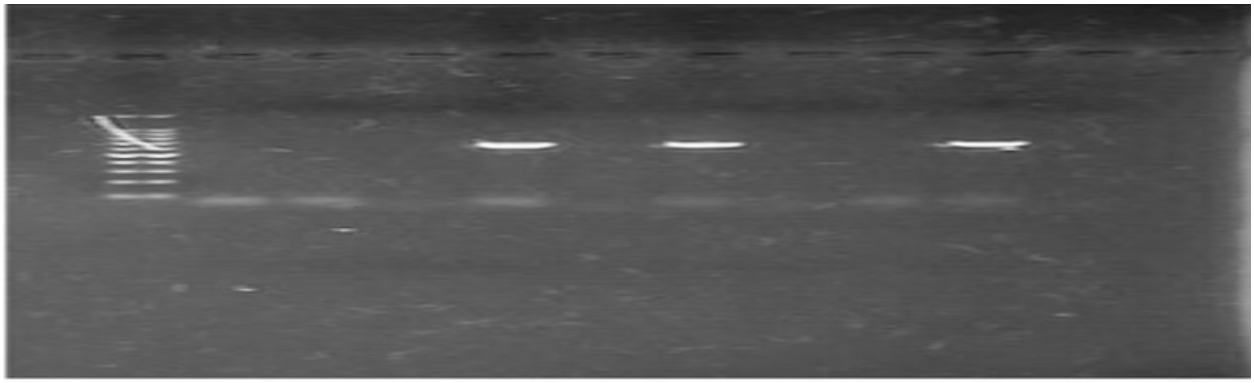

FIGURE 2. Exploration of ***cvcC*** gene in isolates (1-10). (+) isolates give line or stripe (680 basepair); lane M: 1Kb Ladder of DNA ; lanes 4,6,9 were (-) isolates.

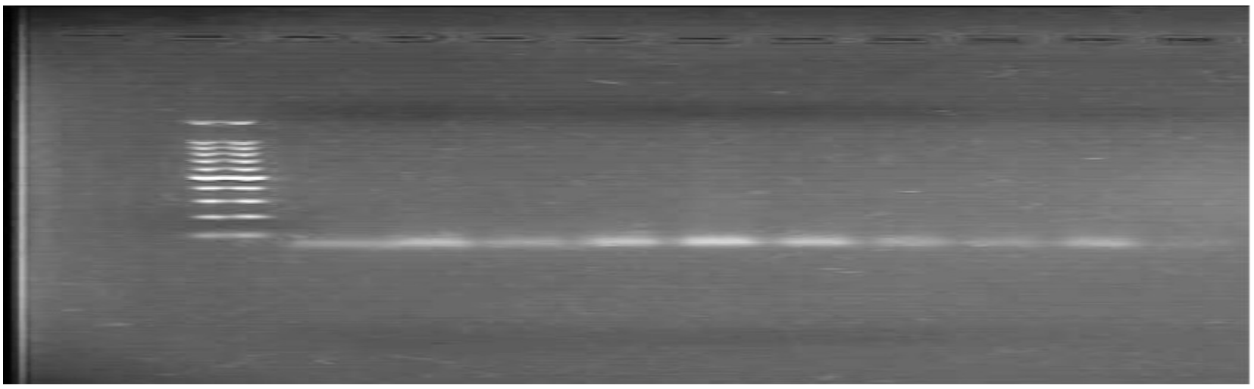

FIGURE 3. Exploration of ***iss*** gene in isolates (1-10). (+) isolates give line or stripe (270 basepair); lane M: 1Kb Ladder of DNA; lanes were (-) isolates.

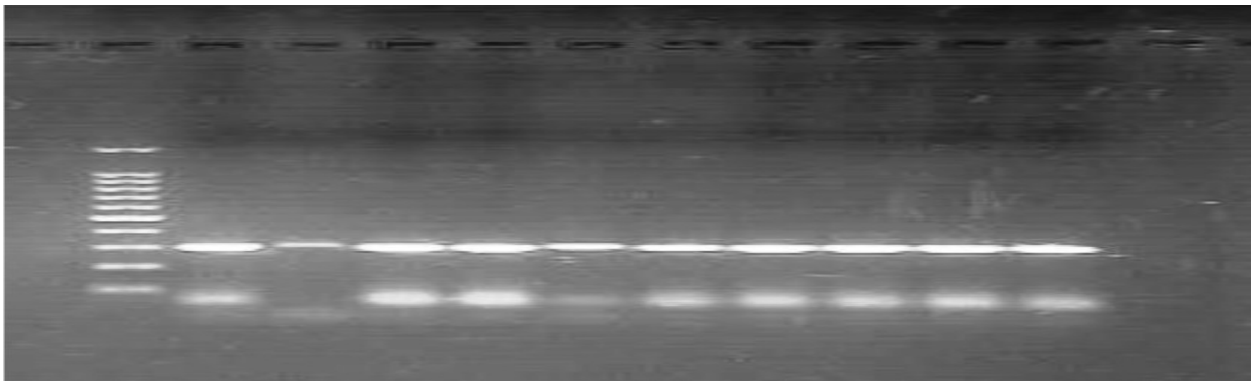

FIGURE 4. Exploration of ***iutA*** gene in isolates (1-10). (+) isolates give line or stripe (300 basepair); lane M: 1 Kb Ladder of DNA ; all lanes were (+)isolates.

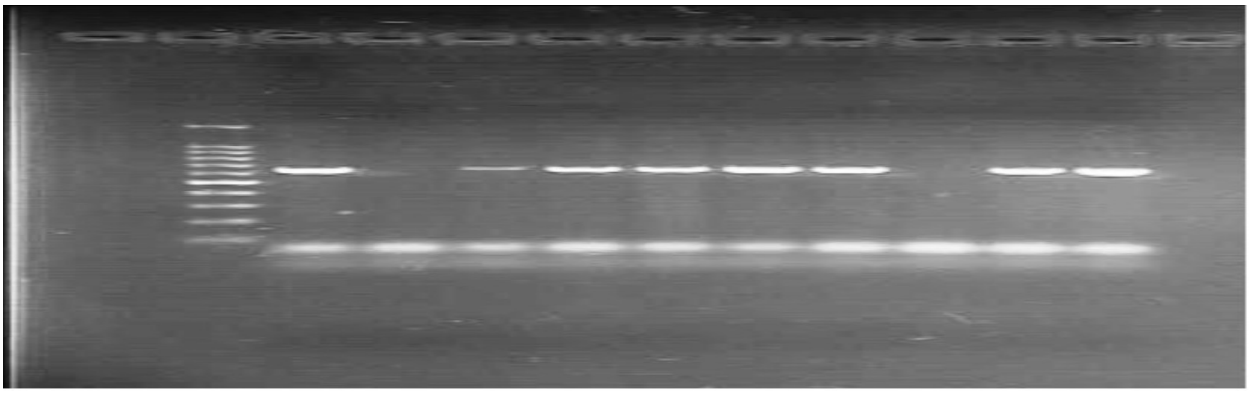

FIGURE 5. Exploration of ***tsh*** gene in isolates (1-10). (+) isolates give line or stripe (620 baispair)); lane M: 1 Kb Ladder of DNA ; all lanes were (+) isolates but isolates number two & eight.

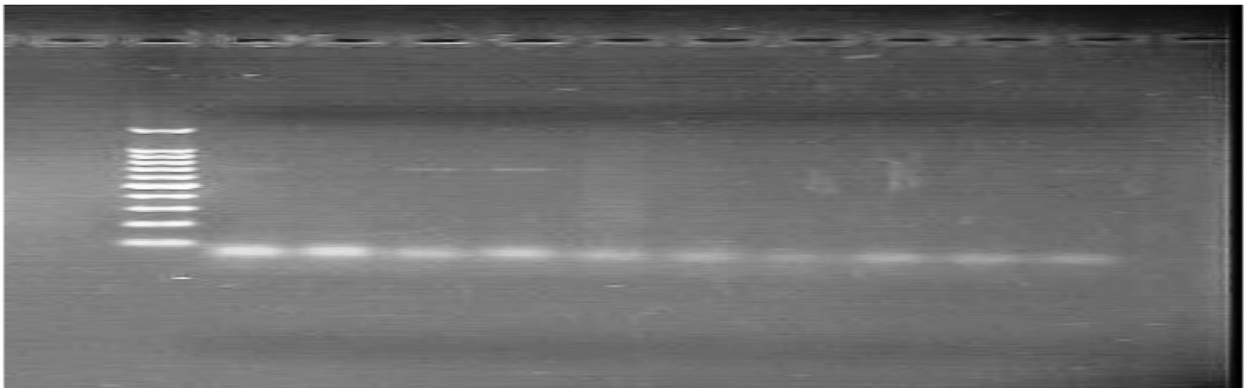

FIGURE 6. Exploration of ***kpsII*** gene in isolates (1-10). (+) isolates give line or stripe (272 basepair); lane M: 1Kb Ladder of DNA ; lanes were (-) isolates.

Conclusions

Only three of the virulence-link six genes were distinguished in different percentages in the study isolates. In three APEC isolates, no genes were distinguished , which makes these strains less virulent than others. Results of our study emphasized the results of the researchers declared about the subject of our research, when looking at the search results , the detected pathogenic *E. Coli* strains had not all the virulent 6 genes individually as well as collectively and that isolated strains from cases of colibacillosis are considered pathological strains even if they don't contain pathological genes. The result of this description (virulence ,causes of virulence, its terms of E. coli) supports the multigenic determination of APEC strains virulence discovered not only by the existence of genes, but by the association of them also. Strains of APEC had presented pathological properties and shelter for the virulence genes which provide a threat to the health of poultry and people. The different & diverse putative pathogenic genes determined in all isolates revealing that infection with E. coli was multi-factorial and multi-faceted which maybe an origin interest in the diagnosis and take in infections control due to APEC. Although of the virulence genes relationship, that existed in our study and in the related articles, had proposed, the actual interplay of the factors of pathogenic in APEC , But in spite of the scientific and technical improvement, the participation to their virulence has not been established so far. The high level of restrictions of antimicrobials utilization in poultry production world ,didn't not let professionals being know on the pathogenic E. coli possibilities to get the infection , to control their transactions, to do this correctly, new procedure is required to disclose the virulence of isolates in an efficient objective and fast way. Multiple pathways had been showed by *E. Coli* strains to pathogenicity that calls attention the risk required by these microbes to the poultry. The embarrassment to the public health of this is also immense due to a possible continual contact of humans to poultry & chicken manure. Also chicken *E. Coli* multi-drug resistant had been reported in study area [48], because having these antimicrobial resistant genes then this will give a chance to the hypothetical danger to the human and poultry health [49]

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