

**In investigation of the gene responsible for the production of *hlyA* enzyme and *aggA* gene in *E. coli* isolated from the diarrhea of chicken by polymerase chain reaction in the province of Al-Qadisiyah**

التحري عن المورثة المسؤولة عن إنتاج إنزيم *hlyA* ومورثة *aggA* في الأشريشيا القولونية المعزولة من اسهال الدجاج بواسطة تفاعل البلمرة المتسلسل في محافظة القادسية

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**Summary :**

This study aimed to know the prevalence and occurrence of *hlyA* gene and *aggA* in *E. coli* that taken from clinical Diarrheic in Chickens by using polymerase chain reaction (PCR) technology in AL-Qadisiyah Province , 55 of fecal samples has sent to microbiological lab to make microbiological culture and PCR assay, extraction DNA as the first step, run the extracted samples in thermocycler apparatus by using primers design online as the second step , then immigration the samples by using electrophoresis to read the results .(72.72%) represents percentage of isolated *E. coli* in our study by use culture methods on MacConkey agar, Eosin methylene blue agar and CHROMagar salmonella, also prevalence of *hlyA* and *aggA* gene in present study were (45%) and (37.5%) respectively , the virulence strains have more resistance than non- virulence stains, *hlyA* gene and *aggA* gene in *E. coli* contributes in the pathogenesis of organism.

**Keyword :** *Escherichia coli* , *hlyA* gene , *aggA* gene , Chickens.

**الخلاصة :**

هدفت هذه الدراسة الى معرفة انتشار وحدث مورثة *hlyA* و *aggA* في الاشريشيا القولونية التي أخذت من الإسهال السريري في الدجاج باستخدام تقنية تفاعل البلمرة المتسلسل في محافظة القادسية . ارسلت 55 عينة براز إلى مختبر الأحياء المجهرية لإجراء زرع الأحياء المجهرية واستخلاص الحامض النووي كخطوة أولى ، مباشرة العينات المستخلصة بواسطة جهاز الدوار الحراري بواسطة استخدام بادئات صممت على شبكة الانترنت كخطوة ثانية ، بعدها ترحيل العينات باستخدام جهاز الترحيل الكهربائي لقراءة النتائج ، (72.27 %) تمثل النسبة المئوية لجراثيم الاشريشيا القولونية المعزولة في دراستنا وبواسطة استخدام طرق الزرع على وسط الماكونكي ، وسط الايوسين مثلين الازرق و وسط كروم السالمونيلا ، كما كان انتشار مورثة *hlyA* و *aggA* في الدراسة الحالية (45%) و (37.5%) على التوالي ، العتر الضارية تمتلك مقاومة اكثر من العتر الغير ضارية ، مورثة *hlyA* و مورثة *aggA* في الاشريشيا القولونية تساهم في امراضية الكائن . الكلمات المفتاحية : الاشريشيا كولاي ، مورثة *hlyA* ، مورثة *aggA* ، الدجاج.

**Introduction:**

Colibacillosis is the major that most distributed diseases threatening of poultry [1, 2]. Colibacillosis in chickens is any infection caused partly or entirely, local or general by *E. coli* isolates [3] , Avian colibacillosis causing systemic disease in poultry like enteritis and pneumonia are termed avian pathogenic *E. coli* [4]. Many of strains were more virulence from other strains because it has a virulence genes like *hlyA* gene and *aggA* gene...etc [5] the virulence genes like Dogtooth, it helps the bacteria to investment all nutrient substance that present around [6].

Alpha-hemolysin (*hlyA* gene) is an exotoxin made by some strains of *E. coli* that help the bacteria to invade the tissue during disease induction, its secreted from the organism in blood media to help the lysis of blood and take the iron as a nutrient element [7].

*hlyA* gene encoding protein called alpha-hemolysin, it is a virulence factor, associated with pathogenic *E. coli*, This hemolysin protein belongs to a family of proteins have activity outside the bacteria [8,9]. *aggA* gene is virulence gene, encoding the aggregative adherence fimbria, it has a great role in adhesion to epithelial cell [10].

In addition, the gene *aggA* was detected from *E. coli*, this gene helps the bacteria to attach to membrane tissue as the first step to infection occurrence by encoding the aggregative adherence fimbria [11,4].

Many of European countries that suffer from increasing occurrence of colibacillosis in chicken that induce great economic loss that mostly associated with this disease [12,13] and [14]. Europe not alone, also South America suffer from that, as many studies recorded the occurrence of *aggA* gene and *hlyA* gene associated with most virulence outbreak in human and poultry in Brazil. [15,16,17]

Without these virulence genes, the bacteria can't resist immunological reactions and can't stay for a long time, therefore this study focusing on the detection of some important virulence factor genes (*hlyA* and *aggA* gene) that causes a serious problem related to animals' productions.

## **Materials and methods:**

### **Sampling preparing :**

Faecal samples were taken from 55 diarrheic chickens from the different farm organised and different households in AL-Qadisiyah Province. The samples were taken from cloaca by using clean swabs, then sent to a laboratory for bacteriological investigation.

All fecal samples primarily were grown in nutrient broth at 37°C for (18-24) h, then cultivated on MacConkey agar. After overnight incubation at 37°C, the pink colonies were picked up and subcultured on Eosin methylene blue media to observe the characteristic metallic sheen, then for more identification, the isolates were subcultured on CHROMagar salmonella plate, blue colonies appear on the surface of plate, the well separate colonies were transferred to nutrient agar plate to obtain pure cultures that subjected to standard morphological identification (size, shape, surface texture, colour...etc), for more identification of isolates, they are some biochemical tests such as indole, Catalase production, citrate utilization and Motility test, biochemical reactions which carried out according to [18].

### **Bacterial genomic DNA extraction:**

DNA extraction of *E. coli* was done by using kit called (Presto™ Mini gDNA Bacteria Kit. GeneAid. made in USA). The process was done by adding 1ml of bacterial inoculum that growing overnight in Brain Heart Infusion broth then centrifuge at 10000 rpm for 1 minute, After that, the supernatant was left and extracted bacterial cells pellets were done in another step the extraction was done depending on company directions. After that, the final product was tested by Nanodrop spectrophotometer to confirm its concentration, then kept at -20°C at freezer for performing PCR thermocycler.

**Polymerase chain reaction (PCR):**

PCR assay was done for determination some virulence factor genes and by using a specific primer that designed in this study by using NCBI-GenBank and designing primers by primer3 plus online (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). The primers were chosen (Bioneer Company Korea) and listed in table (1).

Table (1): sequences of primers with size of targeted products of the PCR to the *E coli*

Primer	Sequence (5' - 3')		Product size
EHEC( <i>hlyA</i> )	F	AGCGTACGTTCCGCTGGCAA	696 bp
	R	ACCCGCTGCAGCTTTTGTTCCT	
EAEC( <i>aggA</i> )	F	TGGGCGCAAACGTAACGACG	148 bp
	R	GCGCCATCAATGTCGGGTTGT	

PCR master mix prepared by using kit of AccuPower® PCR PreMix .From company Bioneer-Korea. Premix tubes of the PCR contain freezer-dried pellet to (dNTPs 250µM ,Tris-HCl (pH 9.0),Taq DNA polymerase 1U, 10mM, KCl 30mM, stabilizer ,MgCl2 , tracking dye, and1.5mM) prepared of the PCR master mix depend on the kit of instructions with 20µl total volume ,added 5µl of genomic DNA was purified and also added 1.5µl of 10p mole of forwarding primer with same of reverse primer, premix tube of PCR with PCR water (20µl) and mixed by use vortex. The Thermocycler by setting up the following thermocycler instructions; according to table (2).

Table (2) Thermocycler instructions of PCR

The stage	temperature	The period	repeat
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 s	30 cycle
Annealing	58 °C	30 s	
extension	72 °C	30 s	
Final extension	72 °C	10 min	1

**4. Results:**

**4.1. Isolation and identification *E coli*:**

Out of 55 fecal samples , 40 were positive for *E coli* isolates the prevalence of *E coli* in the fecal sample was (72.72%), All *E. coli* isolates bright pink on MacConkey media (fig 1) [19] and characterized by metallic sheen on EMB media (fig 2) [19] on salmonella CHROM agar the colonies characteristic in rounded and blue fig (3) that checked by further biochemical tests for confirmation the isolates see table (3).

Table (3): biochemical tests results of *E.coli*.

Biochemical test	<i>E.coli</i>
indol	+
catalase	-
Urease test	-
Citrate utilization	-

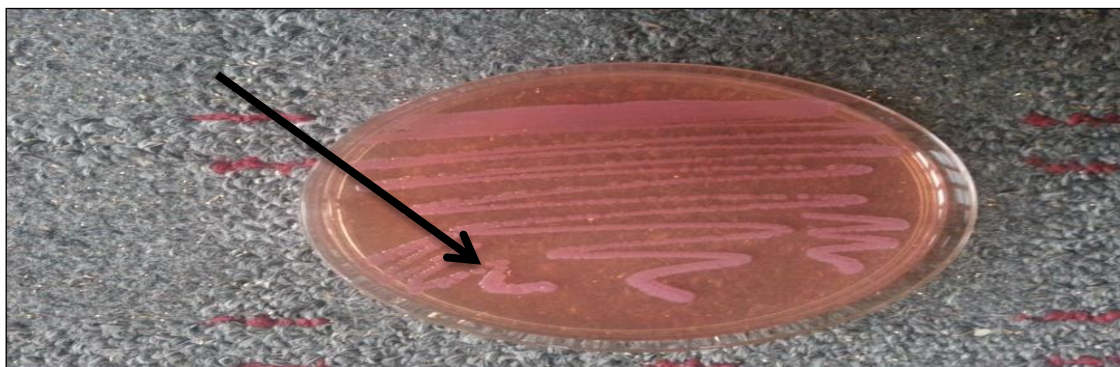


Figure (1): *E.coli* colonies on MacConkey agar (bright pink colonies).



Figure (2): *E.coli* colonies on EMB agar (Metallic sheen).

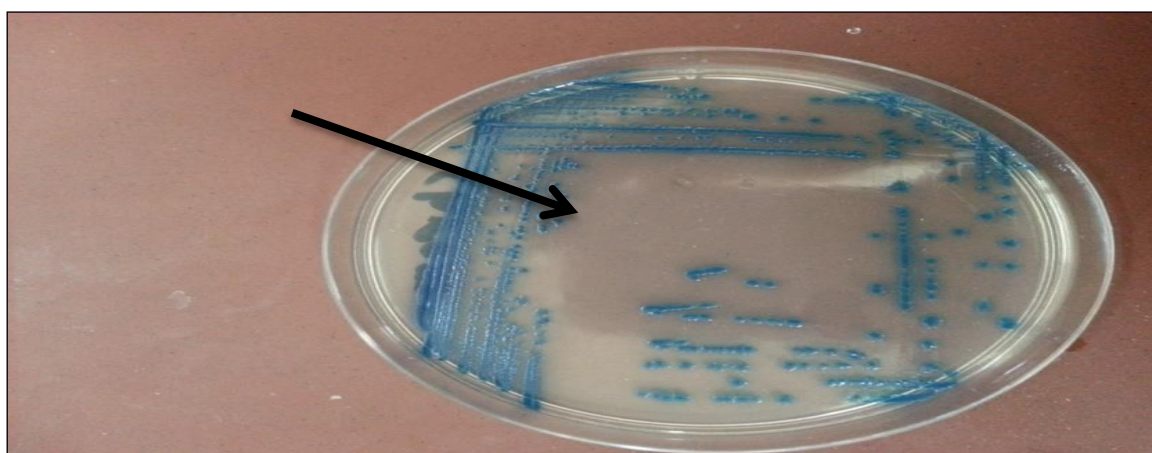


Figure (3) : *E.coli* colonies on CHROMagar salmonella (rounded and blue)

**4.2. Detection of *hlyA* gene in ( Enterohemorrhagic *E.coli* ) by PCR:**

Prevalence of the *hlyA* gene in (EHEC) isolated from diarrhoea chicken, a total of (40) PCR used to analyzed of *E. coli* isolates and the results are summarised in Table (3). Overall, 18 of the 40 (45%) carried gene of *hlyA* gene in (EHEC) diarrhetic chicken, The PCR technique was shown sensitive and specific results when using *hlyA* primer designed by this study using NCBI-Gene Bank and primer 3 plus online by chosen specific *hlyA* primer at PCR product size 696bp (Fig 4).

Table (3): Enterohemorrhagic *E.coli* (*hlyA*) in diarrhetic chicken.

No. of isolates	PCR + ve results	Percentage (%)	PCR - ve results	Percentage (%)
40	18	45%	22	55%

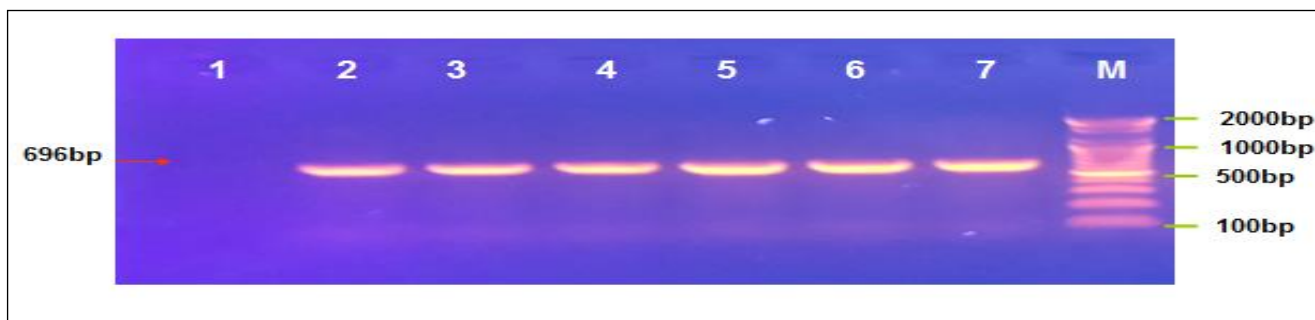


Figure (4): Agarose gel electrophoresis image that shows the PCR product analysis of *hlyA* gene in EHEC (*hlyA*) that isolation from stool Chicken samples. Where M: Marker (100-2000bp), lane negative samples, and lane (2-7) positive PCR samples at 696bp product size.

**4.3. Detection of *aggA* gene in( Enteroaggregative *E coli*) by PCR:**

The Prevalence of Enteroaggregative *E coli* (*aggA* gene) isolated from diarrhoeic chicken, a total of (40) *E.coli* isolates were analyzed by PCR and the results are summarised in Table (5). Overall , 15 of the 40 (37.5%) carried *aggA* gene of (EAEC) diarrhetic chicken, The PCR technique was shown sensitive and specific results when using *aggA* primer designed by this study using NCBI-Gen Bank and primer 3 plus online by chosen specific *aggA* primer at PCR product size 148bp (Fig 5 ).

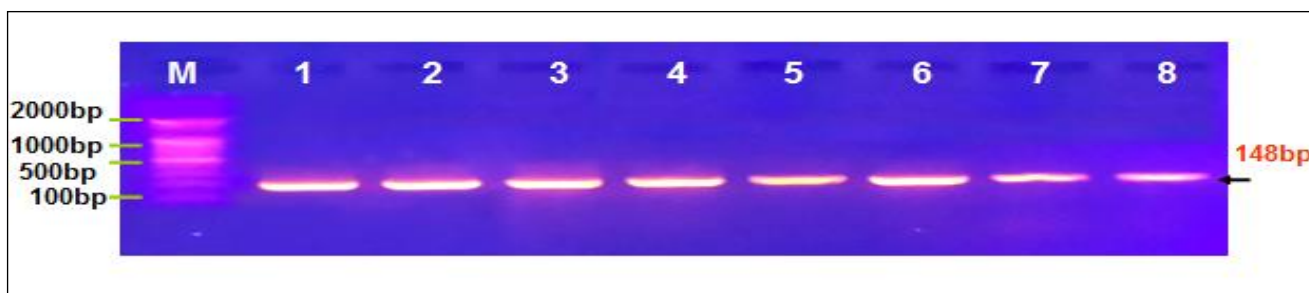


Figure (5):Bands on Agarose gel electrophoresis that represent PCR product of Aggregative adherence fimbriae *aggA* gene in EAEC(*aggA*) positive isolates: Where, Lane (M) DNA marker (100-2000bp), Lane (1-8) positive samples at 148bp PCR product size at 1% Agarose , 100 volt and 80am for 1hour.

Table (5): Enteroaggregative *E.coli aggA* gene in diarrhetic chicken.

No. of isolates	PCR + ve results	Percentage (%)	PCR - ve results	Percentage (%)
40	15	37.5%	25	62.5

**Discussion:**

Were depended on colony morphology as it was appeared with pink color when cultured on MacConkey media, and with green when culture on EMB agar, and also depended on biochemical tests and. metabolic activity [20 , 21 , 22]

According to the preset work , the percentage of *E.coli* that isolated from clinical cases of diarrhoea in chicken was (72.72%), that has little differences or near to results of (20) as they were where recorded percentage of (80%) which isolated from the intestine of the chicken using PCR technique in the same way they differente with [23] and [24] as the percentage of isolation were (7.5%) and (24.3%) in chicken respectively, that less than our results. Also [25] found a very low percentage (1.74%) when compere with this study

In contrast [26] and [27] recorded prevalence rate more than this study, where the Mortality percentage may reach even more than (94%) in an outbreak of colibacillosis disease in chickens.

Also the season, the age, warm or cold environment area play a great role in the prevalence of where, the disease diffuse in winter more than summer, small animals show more susceptibility to

infection than adults , also there are grossly differences in percentage of occurrence of *E.coli* strains between cold and warm region [28] .

The result of this study found that prevalence of *hlyA* gene that extracted from *E.coli* isolates in clinical cases in chicken is (45%) which corresponded with the results of [29] in Egypt, as they recorded (50.2%) as prevalence.

Although [30 , 31] and [15] in different area, showed a low prevalence value of (*hlyA*) (33%), (25.3%) (20.9%) respectively, as compared with the present results , Many other studies recorded higher prevalence rate of *hly A* gene in *E.coli* [32] and [33] as they recorded (71.3%) in Canada, and (77%) in USA respectively.

According to this results, *aggA* gene was detected in *E.coli* isolates in percentage of (37.5%).However, a nearly proportions of *aggA* (41%, 32%) were shown by [34 and 35] respectively ,while [36] found a high prevalence of *aggA* gene in *E.coli* isolates, it was (61%). it is more than our results.

Geographical variation, type of strain, mutation status and virulence of the strain may be interfering with incidence the virulence genes [37].

Some studies suggested *E.coli* have high adaptation within tropical, subtropical area and it has ability to develop and update genetic material by enquired and alteration of the virulence genes that included in the plasmid or DNA [38,39].

In conclusion, the current study confirms the presence of virulent factors in *E.coli* isolate in chickens that will have a negative effect and hazard on the health of the humans when contaminated chicken and eggs have consumed.

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