Molecular Identification of *E. coli* Virulence Factors Genes, *bfp* and *elt*, in Feces of Diarrheic Chickens

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Abstract

The study was aimed to demonstrate the existence of major virulence factors *bfp* and *elt* genes for enteropathogenic *E. coli* (EPEC) and enterotoxogenic *E. coli* (ETEC), respectively, in feces of diarrheic chicken by using PCR technique. For this purpose, 50 diarrheic chickens were subjected for fecal samples collection. In first, the samples have been cultured on MacConkey and Eosin Methylene Blue agars, and the isolates were tested by some specific biochemical tests to verify of *E. coli* samples, which revealed on 20 positive samples. The twenty positive samples were submitted, only, for PCR technique to confirm the existence of *elt* and *bfp* genes. With genes detection, the overall results of the present study were showed that 11/50 (22 %) and 7/50 (14 %) were positives for EPEC-*bfp* and ETEC-*elt* genes, respectively. Also, the cross-infections results with *bfp* and *elt* genes had been identified that 5/20 (25%) were positives with both genes, 2/20 (10 %) were positives with *elt* gene, only, and 6/20 (30%) were positives with *bfp* gene, only. Statistically, the significant differences were reported between the prevalence rates of *bfp* and *elt* genes, at a level of $P \leq 0.05$.

Keywords: *E. coli*, *bfp*, *elt*, Molecular identification, Virulence factors, Diarrheic chicken

Physiology Classification QR1-502-75-9905

Introduction

Escherichia coli are enteric bacteria that can be found in intestinal tracts of human, poultry and animals, where they survive as natural inhabitants without any harm toward human body system [1]. As well as, it considers as one of the most common bacteria that, usually, can be found in soil, water and food due to contamination from feces or during slaughter [2]. Nevertheless, some strains of *E. coli* are pathogenic and have a highly great potential risk to resulting with health problems to humans due to food and water poisoning [3]. Several *E. coli* classes have been recognized such as enteropathogenic (EPEC), enterotoxogenic (ETEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC) and enteroaggregative (EAEC) [4]. EPEC is one of most patho-types that produce a potentially infant diarrhea and responsible on food poisoning outbreaks at developing nations such South Korea, Japan and Australia [5], [6]. EPEC can produce their infecting (attach and efface) lesions by the adhesion with enterocytes and wrecking of microvilli [7]. The mechanism of initial binding to host epithelial cells mediated by a type IV pilus, with a major structural subunit encoded by bundle forming pilus (*bfp*) gene that persistence in adherence plasmid of EPEC [8]. Whereas, the enterotoxogenic *E. coli* (ETEC) infection can occurs due to ingestion of contaminated food or water to produce an abdominal cramps, low-grade fever, watery diarrhea, and nausea [9]. Mainly, two enterotoxins are produced by ETEC involved heat stable and heat labile toxins that play a distinct role in pathogenesis of infections [10]. The heat stable can be divided, also, into two groups of toxins are methanol-soluble protease resistant (STI) and methanol-insoluble protease sensitive (STII) that different structurally, functionally and genetically [11]. However, the gene of heat labile toxin is encoded by the (*elt*) operon that, probably, the most well characterized virulence determinants of ETEC because of its close homology with cholera toxin [12]. In several studies, many techniques, in which PCR, have been applied and targeting of specific virulence factors- genes detection [4], [13] [14]. PCR was revealed a high sensitivity, specificity and simplicity in amplification and identification of specific genes in chickens [15]. Hence, the goal of present study was to detect the specific virulent factor EPEC *bfp* and ETEC *elt* genes

isolated from diarrheic chickens through an application of PCR technique.

Material and Method

Samples obtaining and preparation: An overall 70 fecal samples was obtained from diarrheic chickens of a farm in Al-Qadisiyah province / Iraq, in 2016. These samples were transported to the laboratory in Phosphate Buffered Saline (PBS) transport mediums.

Culture of fecal samples: A full loops of samples were planned on Mac Conkey agar (CDH/India), which incubated (24h/ 37°C), and then, the pink colonies were sub-cultured on Eosin Methylene Blue agar (EMB) (SRL/India) and incubated for 24 hours at 37°C. The colonies that appeared as green metallic sheen were isolated for a further confirmation by a set of biochemical tests (Lactose-fermentation, Indole-production, Simmon- citrate) [16].

DNA Preparation: Subsequently, a sweep of some colonies were inoculated in Luria-Bartani (LB) broth (Himedia/India) and incubated overnight at 37°C, which lysed after that by adding of Sodium Dodecyle Sulfate 0.5 % (Sigma-Aldrich / India) and Proteinase K 100 µg/ml (Sigma-Aldrich / India). After treatment with Cetyltrimethylammonium Bromide (Sigma-Aldrich / India) (in presence of sodium chloride 0.7), and subsequently with Chloroform-Isoamyl alcohol 24:1 (Sigma-Aldrich / India) and Phenol-Chloroform-Isoamyl alcohol 25:24:1 (Sigma-Aldrich / India), the DNA solution was precipitated with 0.6 volume of Isopropanol, and then rinsed with Ethanol 70% and re-dissolved in 100 µ of 10 mM Tris-Hcl (pH=8) with 1mM -EDTA. The DNA solution was diluted 10 folds with sterile water and the diluted DNA solution was used for PCR [17], [18].

PCR and Primers: According to manufacturer's instruction (Sigma-Aldrich / India), the PCR reaction for amplification of bfp and elt genes was done in standard 25 µl reaction in 0.2 ml PCR tube, separately, and 2 µl of DNA was used as atemplet. Twenty (20) picomolar solution of each primer, 1X Taq amplification buffer, 1mM $MgCl_2$, 200 µM each nucleotides and 1.25 units of Taq DNA polymerase, were used for each reaction. The mixture containing PCR tubes were quickly spun at 10000 rpm for 1 minute and placed in thermal

cycler (Germany). The cycling conditions for amplification involved an initial denaturation (30 cycles at 94°C for 1 minute), an annealing temperatue (59°C for 1 minute), and polymerization (at 72°C for 1 minute) that followed by final extension (at 72°C for 10 minutes) [19]. The primers were used to detect two different E. coli virulence genes included (EP1 [5′-AATGGTGCTTGCGCTTGCTGC] and EP2 [5′-GCCGCTTTATCCAACCTGGTA]) for EPEC (*bfp*) gene [14], whilst, (ST1 [5′-TAGAGACCGGTATTACAGAAATCTGA] and ST2 [TCATCCCGAATTCTGTTATATATGTC]) for ETEC (*elt*) gene [4]. The positive amplification was visualized by electrophoresis of the product size at 1 % agarose gel stained with ethidium bromide, at 331 bp for EPEC *bfp* gene (Figure 1) and 583 bp for ETEC *elt* gene (Figure 2).

Statistical analysis: All received results, related to positive and negative diarrheic chickens with *E. coli* by cultures and with confirmatory biochemical tests, were tabled by using a computerized Microsoft Office Excel (2013). Also, the differences and relationships related to both virulent factors genes, EPEC (*bfp*) and ETEC (*elt*), isolated by PCR technique were analyzed, statistically, by using a computerized IBM/SPSS program at level of P≤0.05 [20].

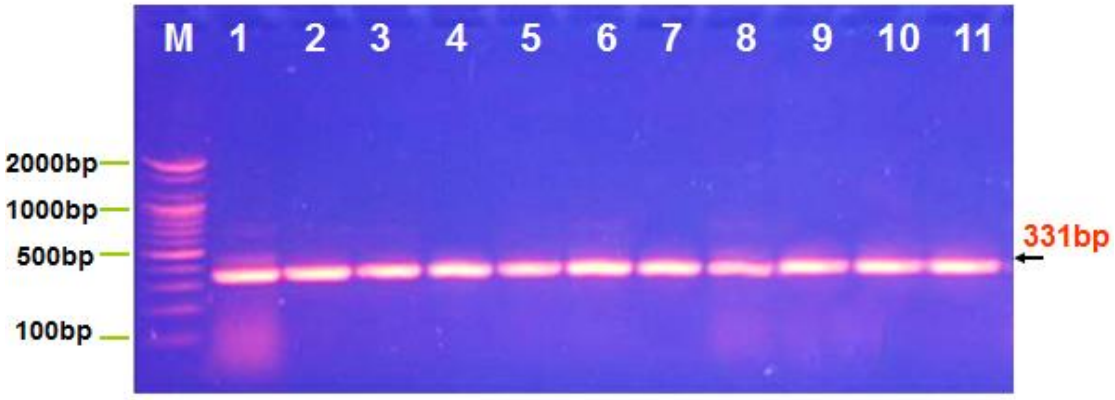

Figure (1): Agarose gel electrophoresis of bfp gene specific for virulent factor, bundle-forming pili, in EPEC positive isolates
Lane (M) DNA marker (2000-100bp), Lane (1-11) positive samples at 331bp

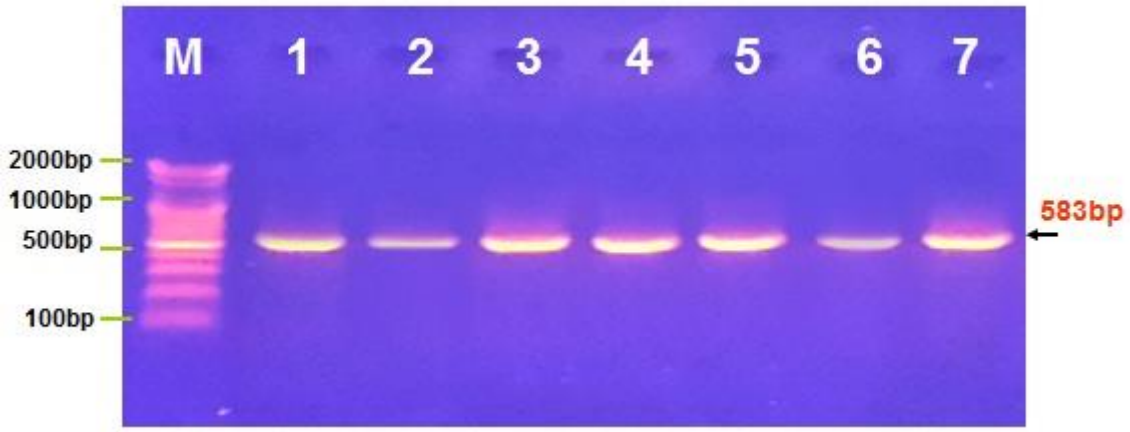

Figure (2): Agarose gel electrophoresis of *elt* gene specific for virulent factor, heat labile enterotoxine, in ETEC positive isolates.
Lane (M) DNA marker (2000-100bp), Lane (1-7) positive samples at 583bp

Results

∴ The result of (Table 1) was showed that in a totally 50 diarrheic chickens submitted for fecal samples collection, the positive *E. coli* isolates by cultures was 20 (40 %).

Table (1): Positive *E. coli* Isolates by Culture Medias

Total samples	Positive samples	Negative samples
50	20 (40 %)	30 (60 %)

∴ In (Table 2), the positive 20 samples by culturing were tested to detect the virulent factors genes, *bfp* and *elt*, of EPEC and ETEC, respectively, by using of a molecular PCR technique.

Table (2): Detection of *bfp* and *elt* genes by PCR

No.	Gene detection	Positive samples	Negative samples
1	EPEC *bfp* gene	11 (55 %) [a]	9 (45 %)
2	ETEC *elt* gene	7 (35 %) [b]	13 (65 %)

Variations in small letters, vertically, referred to a significant difference at level of P≤0.05

The results of (Table 2) revealed that 11 (55 %) of samples were positives with EPEC *bfp* gene (Figure 1), while 7 (35 %) of samples were positives with ETEC *elt* gene (Figure 2).

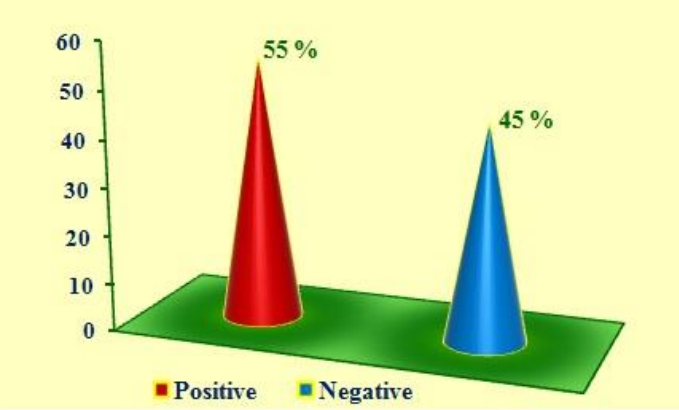

Figure (1): Positive diarrheic samples with EPEC *bfp* gene

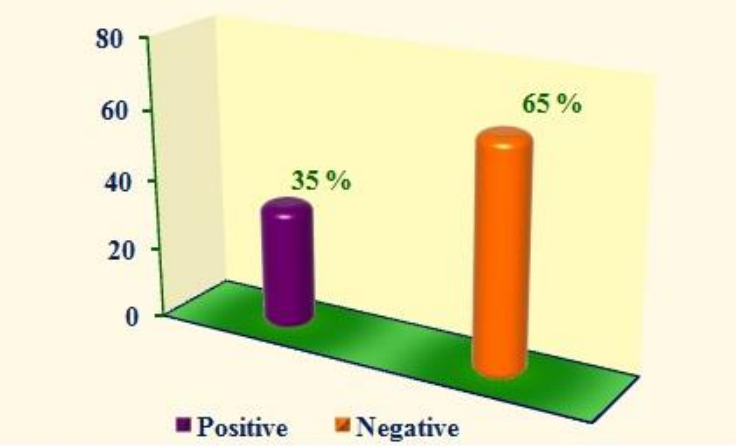

Figure (2): Positive diarrheic samples with ETEC *elt* gene

The prevalence of positive samples with EPEC *bfp* and ETEC *elt* genes in among all samples of study were 11 (22 %) and 7 (14 %), respectively, as in (Table 3).

Table (3): Positive Samples of EPEC bfp and ETEC elt genes in among all Samples of Study

Total Study's Samples	Positives EPEC *bfp* gene	Positive ETEC *elt* gene
50	11 (22 %) [a]	7 (14 %) [b]

Variations in small letters, vertically, referred to a significant difference at level of P≤0.05

∴ In (Table 4): The cross-infections results of virulent factors genes, *bfp* and *elt*, by PCR technique detected that 5 (25 %) positive samples have both *bfp* and *elt* genes, 2 (10 %) positive samples have only *elt* gene; and 6 (30 %) positive samples have only *bfp* gene.

Table (4): Cross-Infections results of virulent factors genes, *bfp* and *elt*, by PCR

	EPEC *bfp* gene results		
ETEC *elt* gene results	Positive	Negative	Total
Positives	5 (25 %) ^{Ab}	2 (10 %) ^{Bb}	7 (35 %)
Negatives	6 (30 %) ^{Ba}	7 (35 %) ^{Aa}	13 (65 %)
Total	11 (55 %)	9 (45 %)	20

Variation in large and small letters, horizontally and vertically, referred to significant differences at level of P≤0.05

Discussion

Escherichia coli are a predominant facultative anaerobic organism that has many strains inhabitant as a flora in digestive system of animals as well human. However, certain strains have been demonstrated to posses the ability for producing a number of virulent factors which captured through the horizontally transferring of genes, and resulting in public-health problems [21].

In Iraq, the prevalence status of ETEC and EPEC in chickens, and their contribution for inducing diarrhea was unknown or uncertain. Hence, the prevalence of EPEC and ETEC with appropriate scale was conducted in this study through determination of *bfp* and *elt* genes in diarrheic chicken by using of a molecular PCR technique. The *bfp* gene was a proved to be more specific for EPEC strains, which proposed to belong to the type IV fimbrial group that including a number of different bacteria such as *Vibrio cholera*, *Neisseria* spp., and *Pseudomonas aeruginosa* [22]. In industrialized countries, EPEC had been connected with several outbreaks of diarrhea in infants, and it's remained a cardinal reason for mortalities in developed countries [23]. EPEC was implicated in cases with gastroenteritis, cystitis, colitis, pyelonephritis, peritonitis and puerperal sepsis as well as food poisoning outbreaks [24]. Interestingly, the *bfp* gene related to EPEC was suggested that it might be play a

wider role in *E. coli* pathogenesis [25]. Like many diarrheal diseases, ETEC is a consequence of inadequate sanitation which is not likely to be resolved in the near-term future in many developing countries [10]. ETEC has pursued a general basic strategy that in usual mediated by a number of proteinaceous virulence factors that in consideration are plasmid-encoded colonization factors. An *elt* is one of these plasmid-encoded factors that investigated by several studies as a major secretory diarrheic determinants for ETEC virulence [26]. Although, both heat labile and heat stable toxins had different mechanisms for action, it's clearly that the production of heat labile toxin might be contributed for diarrhea that cooperated by other toxins [27]. As well as, the cross-infections results reported the probability of persistence both *bfp* and *elt* genes in same samples of diarrheic chickens. Worldwide, the prevalence of *bfp* and *elt* genes was explored from poultry samples, cloaca or meat. However, the prevalence *bfp* and *elt* genes reported, globally, were variable between studies and ranged from 2.3 % to 28 %, and from 0.92 % to 25.2 %, respectively [4], [14]. In slaughtered chickens, *E. coli* act as indicator organisms for unhygienic conditions during processing, handling and distribution; and the presence of it in high numbers indicates that originated from fecal pollution [24]. However, the presence of positive *bfp* and *elt* genes or other virulent factor genes, in same samples, could because of that the animals were harbored for these pathogenic strains of *E. coli*, and this in agreement with results reported previously by [28], [29], [30].

Lastly, the present study's results concluded that the diarrheic chicken samples have been harbored for virulent factors *bfp* and *elt* genes of EPEC and ETEC, respectively. Also, the results reported that the *bfp* gene was more prevalent that *elt* gene. Hence, the further exploration of other virulence factors related to EIEC, EHEC, and EAEC in chickens or other birds because of their hazards on public health.

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التشخيص الجزيئي لجينات عوامل الضراوة ، *bfb* و *elt* ، للايشريكيا القولونية في براز الدجاج المصاب بالاسهال

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الخلاصة

هدفت الدراسة الحالية الى اثبات وجود الجينات الرئيسية *bfp* و *elt* لعوامل الضراوة في الايشيريكيا القولونية المعوية الممرضة (EPEC) والايشيريكيا القولونية المعوية السمية (ETEC) ، على التوالي ، في براز الدجاج المصاب بالاسهال باستعمال تقنية تفاعل البلمرة المتسلسل الجزيئي . لهذا الغرض ، خضعت 50 دجاجة مصابة بالاسهال الى جمع عينات البراز . في البداية ، تمت زراعة العينات على أكاري الماكونكي والايوسين ازرق المثيلين وتم اختبار العزلات مع بعض الاختبارات الكيموحيوية لتأكيد عينات الايشيريكيا القولونية ، والتي كشفت عن 20 عينة موجبة . خضعت العينات العشرين الموجبة ، فقط ، اتقنية تفاعل البلمرة المتسلسل لاثبات وجود الجينين *bfp* و *elt* . مع تشخيص الجينات ، اظهرت النتائج الكلية لهذه الدراسة بأن 50/11 (22 %) و 50/7 (14 %) كانت موجبة للجينين EPEC-*bfp* و ETEC-*elt* ، على التوالي . كذلك ، حددت نتائج الاصابات المتقاطعة مع الجينين *bfp* و *elt* بأن 20/5 (25 %) كانت موجبة مع كلا الجينين ، 20/2 (10 %) كانت موجبة مع جين *elt* ، فقط ، و 20/6 (30 %) كانت موجبة مع جين *bfp* ، فقط . احصائيا ، سجلت الاختلافات المعنوية بين معدلات الانتشار لكلا الجينين ، *bfp* و *elt* ، عند مستوى $P\leq0.05$.

الكلمات المفتاحية : الايشريكيا القولونية ، *bfp* ، *elt* ، التشخيص الجزيئي ، عوامل الضراوة ، الدجاج المصاب بالاسهال