Molecular Detection of the Virulence Genes in *Escherichia coli* Isolated from Healthy and Diarrheic Calves in Southern Iraq

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ABSTRACT

Aim: To investigate the *stx1, stx2, hly, cnf2, sfa/foc, afaI, papC,* and *afaC* virulence-associated genes of pathogenic *Escherichia coli,* isolated from diarrheic and non-diarrheic calves.

Materials and Methods: The genes to be investigated were first isolated from the stool samples obtained from 150 diarrheic and non-diarrheic calves, between the ages of one day and six months, during the period 2016 to 2017. Rectal swabs were aseptically and randomly collected from several herds, from different regions in the province of Basra.

Results of Research: The polymerase chain reaction (PCR) results showed that pathogenic *E. coli* were detected in a total of 34 out of 41 (82.9%) distributed as 23 out of 26 (88.5%) and 11 out of 15 (73.3%) of the diarrheagenic and non-diarrheagenic calves, respectively. The results also showed that *stx1*, *hlyA,* and *stx2* genes showed a higher incidence of distribution in both diarrheagenic and non-diarrheagenic calves, in a percentage rate of 69.6%, 65.2%, 56.5% and 63.6%, 63.6%, 45.5%, respectively. Moreover, eight different virulence gene profiles were established in the present study. Most of the isolates analyzed had at least two or three gene arrangements and only four isolates were seen in a combination of four genes *stx1, stx2, hlyA,* and *afaI.*

Conclusion: It is concluded that *E. coli* isolates from healthy and diarrheic calves that carried various virulence genes, of which the most frequent were *stx1, stx2,* and *hlyA.* A high percentage of these isolates are found in both diarrheic and non-diarrheic calves.

1. INTRODUCTION

In veterinary medicine, diarrhea is considered as one of the most common and critical diseases that causes huge economic losses in neonatal animals. Diarrhea can be caused by bacteria, virus, parasites, and other etiological agents, even as *Escherichia coli* bacteria are being recognized as the leading cause of diarrhea. This microorganism was first described by Theodor Escherichia in 1885. It is a part of the normal gut flora in warm-blooded animals, human beings, and birds. However, some strains are pathogenic and cause different clinical illnesses and diseases (1) *E.coli* also causes septicemia and diarrhea in a wide range of hosts, including man, avian species, and animals such as cattle, piglets, goats, foals, lambs, and buffaloes, as well as children in developing countries, (2) the diarrheagenic form of *E. coli* (DEC) is generally distinguished into Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), Enteroaggregative *E. coli* (EAEC), and adherent *E. coli* (DAEC) (3). Generally, the diarrheagenic form of *E.coli* is known as colibacillosis. Colibacillosis has been linked to clinical symptoms, such as, diarrhea, dehydration, fatigue, fever, malaise, and depression. Calves between three and five days old can also develop colisepticemia, when the bacterial infection spreads into the bloodstream of the host (4,5,6,7). ETEC infection is the most common type of colibacillosis in young animals especially in calves and piglets (8). Moreover, STEC strains are also well-recognized as a cause of colibacillosis in newborn

calves. Even though both healthy and diarrheic calves harbor STEC in their intestine, natural outbreaks and experimental infections have documented the association of STEC with diarrhea and dysentery in young calves (9,10).

Pathogenic *E.coli* can be categorized based on serogroups, pathogenicity mechanisms, clinical symptoms, or virulence factor genes. The pathogenicity of *E. coli* based on virulence factors occurs through plasmids, transposons, bacteriophages, and/or pathogenicity islands (3,11). On the other hand, evidence and information about the *E. coli* occurrence and distribution in neonatal calves in the Basra Governorate is limited based on molecular technique. Thus, it is necessary to develop modern methods or tools to monitor the prevalence of different *E. coli* pathotypes in neonatal calves, in Basra, through the detection of wide-ranging virulence genes. Therefore, in this particular study, the *E.coli* from calves was isolated using the PCR technique and screened through a wide range of virulence genes including the *stx1, stx2, hly, cnf2, sfa/foc, afaI, papC,* and *afaC* genes.

2. MATERIALS AND METHODS

Collection of Stool Samples and Isolation of *E.coli*

E. coli was isolated from rectal stool samples obtained from 150 diarrheic and non-diarrheic calves, between the ages of one day and six months, during the period December 2016 to June 2017. Rectal swabs were aseptically and randomly collected from several herds that were found in different regions in the province of Basra. The samples were collected in sterile screw-capped containers and labeled, to show their serial numbers, as well as time and date of collection. Next, the samples were transported in ice-cooled containers to the Laboratory of Microbiology at the College of Veterinary Medicine, for immediate analysis. The *E. coli* from the samples were isolated and identified using the standard technique (12). All stool samples were inoculated into tubes of freshly prepared Brain Heart Infusion

(BHI) broth and incubated aerobically at 37^{o}C overnight, followed by subculturing on MacConkey agar plates for 24 hours, at 37^{o}C. Of the resulting colonies, the lactose-fermenting colonies were sub-cultured immediately on Eosin methylene blue (EMB) agar and incubated at 37°C for 24 to 48 hours. A colony with a metallic sheen appearance was picked for further analysis using bacteriological and biochemical tests and the API-20E system (bioMerieux, Marcy l'Etoile, France). The bacteriological and biochemical tests included were: Gram staining and culturing on Triple Sugar Iron, Oxidase test, Catalase test, Indole test, and the Urease test, which were carried out for further identification of *E.coli*, according to the previous methods of (12)**.** Following the purification and subculturing on the BHI agar, the isolated bacterial colonies were further identified using the PCR technique.

Bacterial DNA Extraction and PCR analysis

Bacterial DNA was obtained using the boiling method, through the following steps: Five colonies of *E coli* were transferred into Eppendorf tubes containing 100 µl distilled water after being incubated in a BHI agar. The tubes were vortexed and incubated at 100ºC for 20 minutes. Next, 900 µl of distilled water was added and mixed well until the solution was homogeneous. The solution was centrifuged at 12,000 rpm for 10 minutes. The supernatant that contained the genomic DNA was transferred into new Eppendorf tubes, ready for PCR.

The *E.coli* isolates were screened for the following virulence genes: *stx1, stx2, hlyA, cnf, sfa/foc, afaI, papC,* and *afaC,* using conventional PCR, to confirm the identification of *E.coli* isolates on the previously extracted DNA samples, using a master mix (Bioneer, Korea) and a specific set of oligonucleotide primers (Bioneer, Korea), which is illustrated in Table 1. The PCR reaction consisted of: the Master Mix of 5 µl, 1 µl Forward Primer

(Bioneer, Korea), 1 µl Reverse Primer (Bioneer, Korea), and 5 µl DNA Templates. The final volumes of the reaction were adjusted to 20 µl by adding 8 µl of nuclease-free water. The PCR amplification process was accomplished by using a Thermocycler (*Esco*, Singapore), with cycling conditions consisting of initial denaturation at 94^{o}C for two minutes, followed by 35 cycles of amplification (denaturation at 94^{o}C for 30 seconds, annealing for 30 seconds, extension at 72^{o}C for one second, and final elongation at 72^{o}C for seven minutes). The analysis of the PCR products was performed in 2% agarose gel electrophoresis. A 100-bp DNA ladder (Bioneer, Korea) was used as a molecular weight marker and the gels were stained with ethidium bromide, examined, and photographed under ultraviolet (UV) illumination (E-graph – ATTO/Japan). Fragment sizes of approximately 180 bp, 255 bp, 565 bp, 839 bp, 410 bp, 750 bp, 328 bp, and 672 bp, were verified as positive for *stx1, stx2, hly, cnf2, sfa/foc, afaI, papC,* and *afaC* genes, respectively.

Table 1: List of primers, annealing temperatures, and predicted sizes used for the detection of virulence genes of *E. coli* isolates

Target gene	Primers	size of PCR products (bp)	Annealing temperature	Reference
stx1	ATAAATCGCCTATCGTTGACTAC AGAACGCCCACTGAGATCATC	180 bp	60ºC	13
stx2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255 bp	60ºC	13
hly	F-AGATTCTTGGGCATGTATCCT R- TTGCTTTGCAGACTGTAGTGT	565 bp	55 ºC	14
cnf2	GTGAGGCTCAACGAGATTATGCACTG CCACGCTTCTTCTTCAGTTGTTCCTC	839 bp	65 ºC	15
sfa/foc	F:CTCCGGAGAACTGGGTGCATCTTAC R:CGGAGGAGTAATTACAAACCTGGCA	410 bp	58 ºC	16,17
afaI	F-GCTGGGCAGCAAACTGATAACTCTC R-CATCAAGCTGTTTGTTCGTCCGCCG	750 bp	58 ºC	16
papC	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328 bp	63 ºC	18
afaC	CGGCTTTTCTGCTGAACTGGCAGGC CCGTCAGCCCCCACGGCAGACC	672bp	65 ºC	19

Statistical analysis

To demonstrate any association between the results, the exact Fisher test and Pearson's chi-squared test, with Yates correction, were used, with the limit of significance being 5%. Statistical analysis was performed using the SPPS software version 11.5.

3. RESULTS AND DISCUSSION

Prevalence and detection of *E. coli* in rectal swabs collected from calves

Results in Table 2 show the bacterial isolates from the examined rectal swabs of diarrheic and non-diarrheagenic calves. In this Table, a total of 150 rectal swabs were collected from calves (75 diarrheagenic and 75 non-diarrheagenic calves). The samples were cultured on specific and selective media for isolation of *E. coli* and subjected to biochemical tests and the results were confirmed by using the API-20E system. Gram-negative rods were detected by Gram staining, and the isolates produced colonies with a black metallic sheen on the EMB agar and a pink appearance on the MacConkey agar. Therefore, on the basis of bacteriological, biochemical, and API-20E system tests, the *E. coli* was isolated from 26 out of 75 (34.7%) diarrheagenic calves and from 15 out of 75 (20%) non-diarrheagenic calves, with a total percentage of 27.4% (41/150). Additionally, a total of 34 (82.9%) isolates were obtained from the PCR-positive samples and were found to be positive for at least one of the virulence genes as revealed in Table 2. The results of the conventional PCR analysis are shown in Figures 1(a,b,c,d,e,f,g and h).

On the other hand, Table 3 reveals the prevalence of virulence gene distribution among diarrheagenic and non-diarrheagenic calves. In this Table, the *stx1, hlyA,* and *stx2* genes showed a higher incidence of distribution in both diarrheagenic and non-diarrheagenic calves in a

percentage rate of 69.6%, 65.2%, 56.5% and 63.6%, 63.6%, 45.5%, respectively. Moreover, Table 4 shows the virulence gene arrangements in each *E.coli* isolate. It shows that eight different virulence profiles were established in the present study. Most of the isolates analyzed have at least two or three gene arrangements, and only four isolates show a combination of four genes with a profile of *stx1, stx2, hlyA,* and *afaI.*

Table (2): Prevalence of *E. coli* among diarrheagenic and non-diarrheagenic calves based on the API-20E system and PCR detection results.

Type of sample	*E.coli* Isolation based on API 20 E system		*E.coli* Isolation based on PCR results	
	E.coli Isolation no.	*E.coli* isolation(%)	*E.coli* Isolation no.	*E.coli* isolation(%)
Diarrheagenic	26/75	34.7	23/26	88.5%
non-diarrheagenic	15/75	20	11/15	73.3
Total	41/150	27.3	34/41	82.9
P-value	*0.0919		**0.0131	
P-value	***0.0580			

***For all diarrheagenic and non-diarrheagenic samples tested based on API-20E system the differences were not significant ($p > 0.05$)**

****For all diarrheagenic and non-diarrheagenic samples tested based on PCR results the differences were significant ($p < 0.05$)**

*****For all diarrheagenic and non-diarrheagenic samples tested based on API-20E system and PCR results the differences were significant ($p < 0.05$)**

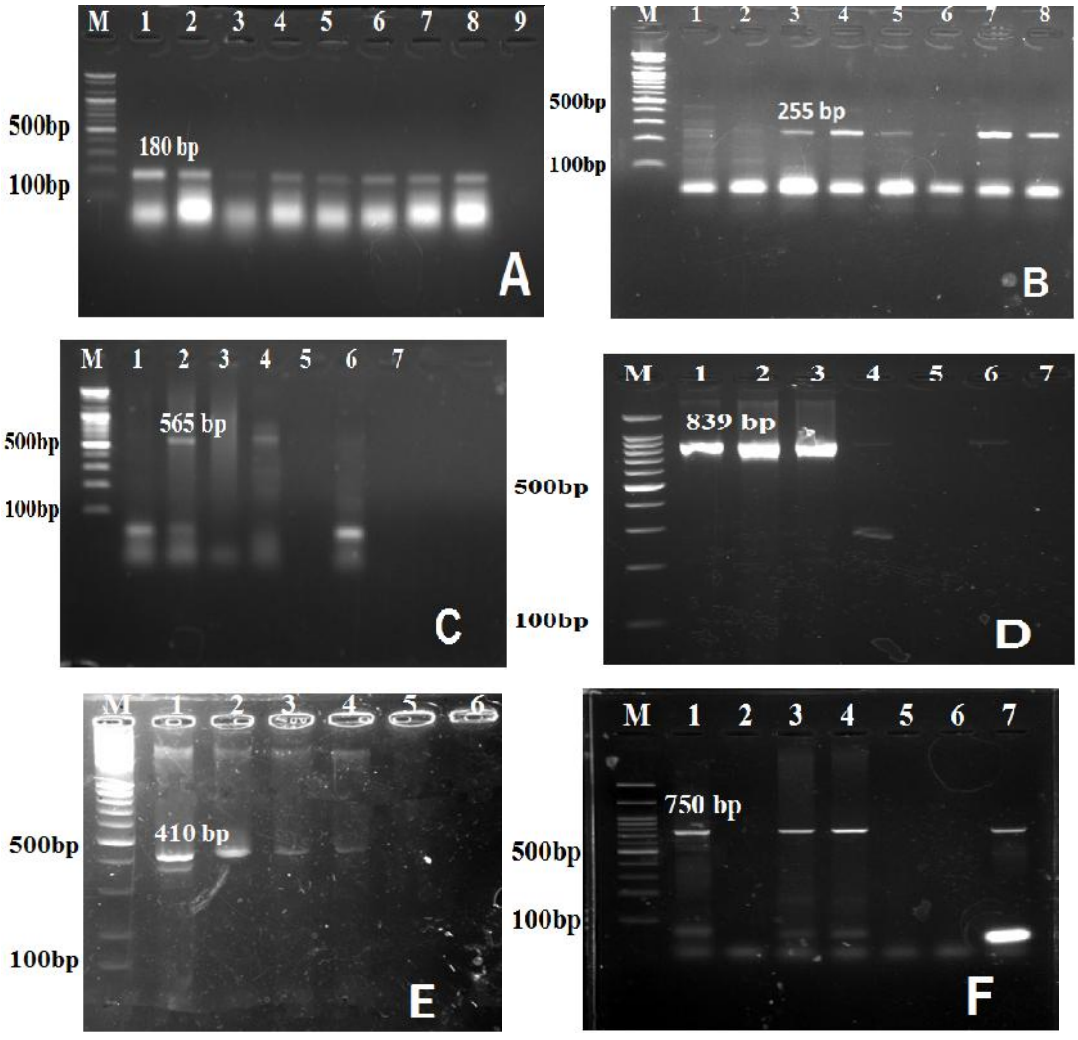
Table 3: Prevalence of virulence gene distribution among diarrheagenic and non-diarrheagenic calves

Virulence gene	Diarrheagenic calves Isolates 23	Non-Diarrheagenic calves Isolates 11	Total 34	P-value
Stx1	16 (69.6)	7(63.6)	23(7.82)	0.0392
Stx2	13(56.5)	5(45.5)	18(52.9)	0.0526
hlyA	15(65.2)	7(63.6)	22(64.7)	0.0684
Cnf	5(21.7)	3(27.3)	8(32.5)	0.7090
Sfa/foc	4(17.4)	2(18.2)	6(17.6)	0.6728
afaI	3(13.0)	1(9.1)	4(11.8)	0.6135
PapC	3(13.0)	3(27.3)	6(17.6)	1.0000
afaC	2(8.7)	1(9.1)	3(8.8)	1.0000

P-value: calculated by exact Fisher's test

Table 4: Virulence gene profiles present in *E. coli* strains isolated from diarrheic calves

No .of genes	Virulence profiles	Number of isolates		Total
		Diarrheagenic calves	Non-Diarrheagenic calves	
1	Stx1,stx2	6	3	9
2	Stx1, hlyA	3	2	5
2	hlyA,Cnf2	2	2	4
3	Stx1,Stx2,hlyA,	4	1	5
3	hlyA,Cnf2, PapC	1	0	1
3	hlyA, Sfa/foc, afaC	2	1	3
3	Cnf2, Sfa/foc, PapC	2	1	3
4	Stx1,Stx2, hlyA, afaI	3	1	4
	Total	23	11	34



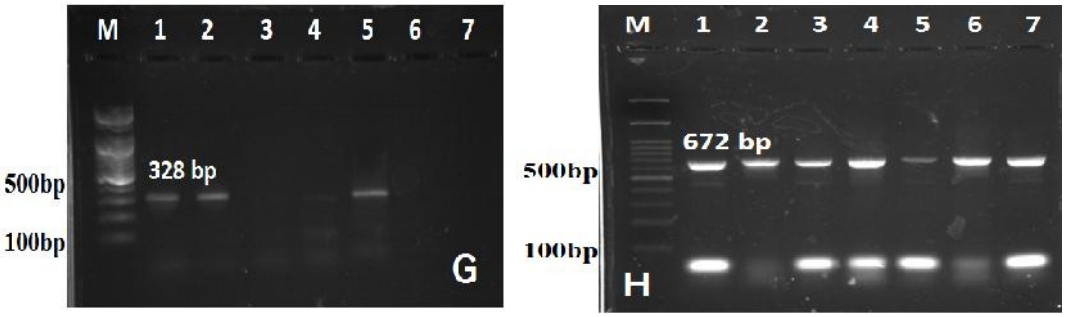


Figure 1. Agarose gel electrophoresis reveals the PCR products of : (A) Lane M: 100 bp DNA Ladder. Lanes 1- 8: PCR Products of 180 bp stx1 gene. (B) Lane M: 100 bp DNA Ladder. Lanes 3,4,5,7 and 8: PCR Products of 255 bp stx2 gene. (C) Lane M: 100 bp DNA Ladder. Lanes (2 and 4) PCR Products of 565 bp hly gene. (D) Lane M: 100 bp DNA Ladder. Lanes(1,2,3,4 and 6) PCR Products of 839 bp cnf 2 gene.(E) Lane M: 100 bp DNA Ladder. Lanes (1-4) PCR Products of 410 bp Sfa/foc gene.(F) Lane M: 100 bp DNA Ladder. Lanes(1,3,4 and 7) PCR Products of 750 bp afaI gene.(G) Lane M: 100 bp DNA Ladder. Lanes (1,2 and 5) PCR Products of 328 bp papC gene.(H) Lane M: 100 bp DNA Ladder. Lanes (1-7) PCR Products of 672 bp afaC gene. All samples were run with control negative

The economy of many developing countries is dependent on the production of livestock animals, which continues to make important contributions to the local food supply. In recent years, animals have been increasingly raised on farms and have spread, particularly in southern Iraq. As a result, several livestock diseases have occurred. Diarrhea in calves, caused by *E. coli*, is one of these diseases, which often occurs as an outbreak and is still one of the most common causes of death in newly born calves (20,21,22,23). Additionally, the findings of the current study are similar to those found in the previous data, which has found that *E. coli* is the most common bacteria isolated from the fecal samples of diarrheic and healthy calves (24). Besides, the presence of pathogenic *E. coli* strains in diarrheic animals is of greater significance because of the high possibility of zoonotic transmission following widespread environmental contamination with these organisms (25,26,27).

In the current study, two diagnostic tools were required API- 20E system and PCR. On the basis of the API -20E system, the prevalence of *E. coli* infection in the examined calves was 27.3%, distributed as 34.7% in calves with

diarrhea and 20% in non-diarrheagenic calves. According to the Table 2 there was no significant difference ($p > 0.05$) between diarrheagenic and non-diarrheagenic calves based on the API-20E system results. On the other hand, the PCR technique was also used to confirm the *E. coli* infection using eight virulence genes, which included *stx1, stx2, hlyA, cnf, sfa/foc, afaI, papC,* and *afaC*. The prevalence of *E. coli* infection in the examined calves was 82.9% (88.5%in calves with diarrhea and 73.3% in non-diarrheagenic calves). With regard to the PCR results there was a significant difference ($p< 0.05$) between diarrheagenic and non-diarrheagenic calves. The explanation of this result is attributed to the fact that the PCR test is more current and specific to detect the pathogenic *E. coli* from clinical and random samples. Moreover, in the current study, seven *E. coli* isolates that were isolated from diarrheic and non-diarrheic samples have no virulence factors. The possible explanation for this finding is the fact that maybe these strains are non-pathogenic *E. coli* and the animals had diarrhea due to some other infectious agents (28).

On the basis of the PCR results, eight virulence genes were used to detect the presence of pathogenic *E.coli* (*stx1, stx2, hlyA, cnf, sfa/foc, afaI, papC,* and *afaC*). Four of them encoded the toxic factors (*stx1, stx2, hlyA,* and *cnf*) and the other four encoded the adhesion factors (*sfa/foc, afaI, papC,* and *afaC*). The prevalence of toxic virulence genes was 23(7.82), 18(52.9), 22(64.7), and 8(32.5) for *stx1*, *stx2*, *hlyA,* and *cnf* genes, respectively. Many previous studies identified that the *stx1* and *stx2* genes were the most toxic genes detected in the *E.coli* from colibacillosis. Studies by (29) were designed to determine the prevalence of STEC and STEC/O157:H7 in healthy sheep.

According to (29), the prevalence of *stx1, stx2, and cnf1* toxic genes in the STEC isolates was higher: 52.2% (47/90), 33.3% (30/90), and 32.2% (29/90), respectively. Similarly, (30,31) observed that 46.5% (20/43) and

55% (213/384) of the STEC isolates carried *stx1*, even as (32) revealed that 17% of all diarrheic and healthy calves of Australian herds were infected by *E. coli,* and 15.2% of the *E. coli* strains harbored the shiga toxin genes including *stx1, stx2, ehly,* and *eae* genes.

The presence of a hemolysin-encoding *hlyA* gene, from the Shiga toxin-producing *Escherichia coli (*STEC) was detected by PCR in each of the 41 strains tested. Fifteen (65.2%) and seven (63.6%) of the *E.coli* isolates from healthy and diarrheic calves, respectively, had the *hly*A gene. This finding is in contrast to a majority of previous epidemiological studies by (33) who developed a multiplex PCR assay for the rapid detection of virulence factor genes in the Enterohemorrhagic *Escherichia coli* (EHEC) in the fecal samples derived from healthy and clinically affected cattle, sheep, pigs, and goats. Hemolysin genes play a critical role in *E.coli* pathogenicity, as they are considered the main factors responsible for cell detachment, and have damaging effects on the epithelial integrity (34,35). Moreover *E. coli* produce several other hemolysin genes. Enterohaemolysin of the enterohemorrhagic *E. coli* (EHEC), designated *Ehx* or *hly* EHEC, is very similar to *h*lyA with regard to its genetic organization and calcium ion dependency, although it is cell-bound (36).

Alternatively, the current results are also consistent with the results found in the study of (37) and (38) where their *E.coli* isolates were PCR-positive for the *hlyA* Hemolysin gene. Similarly, a high occurrence of the *hlyA* gene with *stx1* and/or *stx2* was also detected in the study of (39). The possible explanation for the occurrence of a particular gene combination is the fact that a particular gene is carried by a plasmid and has the ability to transfer among the related bacteria (39). Also, in the current study, the prevalence of the *cnf* gene was low and this finding agreed with the previous studies (40).

According to (40) the distribution of the *cnf* gene in the *E.coli* isolates from diarrheic and healthy animals, and humans, was also detected, but was rare, and the occurrence of the *cnf* gene was not clear, as this gene contributed more to the uropathogenic *Escherichia coli* (UPEC) outbreaks in humans. Hence, the most accepted hypothesis for the existence of the *cnf* gene, which was more related to the necrotoxic *E.coli* (NTEC) strains, was that it occurred as an extraintestinal infection from UPEC and also confirmed the significance of the *cnf* gene isolated from diarrheic and healthy animals as a potential threat to human health (29).

On the other hand, four fimbriae adhesion genes were also detected. The finding of these fimbriae factors in a diarrheal condition is rare, but they are indicators to extraintestinal pathogenic *E.coli* infection (41,42). Moreover, (43,44) they finding the significant association between the occurrence of fimbriae genes detection with strains that produced cytotoxic necrotizing factor that isolated from calves with septicemia or diarrhea. In addition, the Fisher's exact test has also been applied to determine if there is a significant difference between a particular gene occurrence and the calf diarrhea, Table 3. According to Table 3 there is a significant difference ($p = 0.0392$) in the case of the *stx1* gene and no statistically significant difference ($p > 0.05$) in the case of *stx2, hlyA, cnf, sfa/foc, afaI, papC,* and *afaC* genes. This result indicates that the *stx1* gene is related more to the *E.coli* isolated from diarrheagenic and non-diarrheagenic calves and this finding agrees with the finding of (20,45), who detected that the *stx* gene in healthy in diarrheagenic calves

The current study has also revealed eight different combinations of the virulence-associated genes in each *E.coli* isolate. Most of these isolates have at least two or three gene arrangements, and only four isolates show a combination of four genes with profiles of *stx1, stx2, hlyA,* and *afaI*.

However, many researchers have attempted to correlate the existence of recognized virulence profile factors with a disease or severity of a disease and concluded that no single factor is responsible for the virulence of STEC(46,47,78). These variations in gene virulence and severity may contribute to the overuse and misuse of antibiotics in intensive animal production as well as in the crowded and stressful conditions of factory farms. Moreover, the geographical differences and climate conditions also play an important role and influence the shedding of STEC in calves (49,50).

4. CONCLUSION

It is concluded that *E. coli* isolates from healthy and diarrheic calves carry various virulence genes, of which the most frequent are *stx1, stx2,* and *hlyA,* which are present in a higher percentage in isolates from both diarrheic and non-diarrheic calves.

Ethical responsibilities

Protection of human and animal subjects: The authors declare that the procedures followed were in accordance with the Animal Welfare Regulations and Ethics.

REFERENCES

1.Rahman H, Deka M.Detection and characterization of necrotoxin producing Escherichia coli (NTEC) from patients with urinary tract infection (UTI).Indian J. Med. Res. 2014;Apr;139 (4):632-7.

2.Islam1 AK.MA, Rahman M, Nahar A, Khair A, Alam MM. Investigation of pathogenic Escherichia coli from diarrheic calves in selective area of Bangladesh. Bangl. J. Vet. Med. 2015; 13 (1): 45-51.

3.Kaper JB, Nataro JP, Mobley HL. Pathogenic Escherichia coli. Nat. Rev. Microbiol.2004; 2: 123-140.2.

4.Duda-Madej A, Gościniak G, Andrzejewska B, Duda AK, Sobieszczańska B. Association of untypeable enteropathogenic Escherichia coli (EPEC) strains with persistent diarrhea in children from the region of lower Silesia in Poland. Pol J Microbiol. 2013;62:461–4.

5. Multistate Outbreak of Shiga toxin-producing Escherichia coli O157:H7 Infections linked to Ground Beef (Final Update) (2014, June 20) Retrieved September 9, 2014 from www.cdc.gov/ecoli/2014/O157H7-05-14/index.html

6. Mainil J: Facteurs de virulence et propriétés spécifiques des souches invasives d'Escherichia coli: I) les adhésines et facteurs de colonisation. Ann. Med. Vet. 2003; 147:105–126 (in French).

7. Le Bouguénec C, Bertin Y: AFA and F17 adhesins produced by pathogenic Escherichia coli strains in domestic animals. Vet. Res. 1999; 30:317–342.

8. Younis E, Ahmed A, El-Khodery S, Osman S, El-Naker Y. Molecular screening and risk factors of enterotoxigenic *Escherichia coli* and *Salmonella* spp. in diarrheic neonatal calves in Egypt. Res. Vet. Sci. 2009;87:373---9

9.Dean-Nystrom EA, Bosworth BT, Cray WC Jr, Moon HW. Pathogenicity of Escherichia coli O157:H7 in the intestines of neonatal calves. Infect Immun 1997;65, 1842- 1848.

10. Sandhu KS, Gyles CL. Pathogenic Shiga toxin-producing Escherichia coli in the intestine of calves. Can. J. Vet. Res. 2002; 66, 65-72.

11. Nataro JP, Kaper JB. Diarrheagenic Escherichia coli. Clin. Microbiol. Rev. 1998; 11:142–201. [PubMed: 9457432]

12.Quinn PJ, Cater ME, Narkey BK, Carter GR,."*Enterobacteriaceae* ". In: clinic. Vet. Microb.2004; 2nd ed.: 210-236.

13.Paton, Adrienne, and Paton, James C. "Detection and characterization of Shiga toxigenic Escherichia coli by using multiplex pcr assays for stx1, stx2, eaeA, Enterohemorrhagic E. coli hlyA, rfbO111, and rfbO157." Journal of Clinical Microbiology. 1998; 36.2 : 598-602.

14.Mladin C, Usein CA, Chifiric MC, Palade A, Slavu CL, Negut M. Genetic analysis of virulence and pathogenicity features of uropathogenic *Escherichia coli* isolated from patietns with neurogenic bladder. *Romanian Biotechnological Letters*, 2009 (14)6: 4900-4905.

15.Pass MA, Odedra R, Batt RM. Multiplex PCRs for identification of Escherichia coli virulence genes. J Clin Microbiol 2000; 38 : 2001-4.

16.Le Bouguenec C.; Archambaud, M., and Labigne, A. Rapid and specific detection of the *pap*, *afa*, and *sfa* adhesionencoding operons in uropathogenic *Escherichia coli* strains by polymerase chain reaction. *J. clin. Microbiol.*, 1992. 30: 1189-1193.

17.Moulin-Schouleur, M., Re´ pe´ rant, M., Laurent, S., Bre´ e, A., MignonGrasteau, S., Germon, P., Rasschaert, D. & Schouler, C. (2007). Extraintestinal pathogenic Escherichia coli strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. J Clin Microbiol 45, 3366–3376.

18.Soto SM, Zuniga S, Ulleryd P, and Vila J.Acquisition of Pathogenicity Island in an Escherichia coli clinical isolates causing febrile urinary tract infection. Eur J Clin Microbial Infect Dis. 2011;10: 1258-62

19.Le Bouguénec, C., Lalioui, L., du Merle, L., Jouve, M., Courcoux, P., Bouzari, S.Characterization of AfaE adhesins produced by extraintestinal and intestinal human Escherichia coli isolates: PCR assays for detection of

Afa adhesions that do recognize Dr blood group antigens. J Clin Microbiol. 2001;39: 1738–1745.

20. de Moura M, Ludovico GF, Valadares MSV, Gatti DS, Leite C. Detection of virulence genes in Escherichia coli strains isolated from diarrheic and healthy feces of dairy calves in Brazil. Arq. Inst. Biol., São Paulo. 2012; v.79, n.2, p.273-276, abr./jun.

21.Bartels C, Holzhauer M, Jorritsma R, Swart W, Lam T. Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. Prev Vet. Med. 2010;1;93(2-3):162-9.

22.Bendali F, Bichet H, Schelcher F, Sanaa M. Pattern of diarrhoea in newborn beef calves in south-west France. Vet Res. 1999;30:61-74.

23.Morrell E, Moore D, Odeón A, Poso M, Odriozola E, Cantón G, Paolicchi F, Malena R, Leunda M, Morsella C, Campero C. Retrospective study of bovine neonatal mortality: cases reported from INTA Balcarce, Argentina. Rev Argent Microbiol. 2008;40:151-7.

24. Osman1 KM, Mustafa1 AM, Elhariri M, AbdElhamed GS. The Distribution of Escherichia coli Serovars, Virulence Genes, Gene Association and Combinations and Virulence Genes Encoding Serotypes in Pathogenic *E. coli* Recovered from Diarrhoeic Calves, Sheep and Goat. Transboundary and Emerging Diseases. 60 (2013) 69–78.)

25. Geser N, Stephan R, Kuhnert P, Zbinden R, Kaeppeli U, Cernela N, Haechler H. Fecal carriage of extended-spectrum betalactamase-producing enterobacteriaceae in swine and cattle at slaughter in Switzerland. J. Food Prot. 2011; 74, 446-449.

26. Nguyen Y, Speradio V. Enterohemorrhagic *E. coli* pathogenesis. Front Cell Infect Microbiol. 2012; 2:90.

27. Leomil L, Aidar-Ugrinovich L, Guth BEC, Irino K, Vettorato MP, Onuma DL, Castro AFP. Frequency of Shiga toxin-producing Escherichia coli (STEC) isolates among diarrheic and non-diarrheic calves in Brazil. Vet Microbiol. 2003 ; 97: 103–109.

28. Shahrani M, Dehkordi FS, Momtaz H. Characterization of Escherichia coli virulence genes, pathotypes and antibiotic resistance properties in diarrheic calves in Iran. Biological Research. 2014;47(1):28.

29. Ferreira MRA, Silva TDS, Stella AE, Conceicao FR, Reis EFD, Moreira CN. Detection of virulence factors and antimicrobial resistance patterns in shiga toxin-producing Escherichia coli isolates from sheep. Pesquisa Veterinaria Brasileria. 2015; 35(9): 775-780.

30. Ghanbarpour R, Kiani M. Characterization of non-O157 shiga toxin-producing Escherichia coli isolates from healthy fat-tailed sheep in southeastern of Iran. Trop. Anim. Health Prod. 2013; 45:641-648.

31. Blanco M , Blanco J.E, Mora A, Rey J, Alonso JM , Hermoso M, Hermoso J, Alonso MP, Dahbi G, Gonzalez EA Bernardez MI, Blanco J. Serotypes, virulence genes, and intimin types of shiga toxin (Verotoxin)-producing Escherichia coli isolates from healthy sheep in Spain. Journal of Clinical Microbiology. 2003; 41(4): 1351–1356.

32. Herrera-Luna C, Klein D, Lapan G, Revilla-Fernandez S, Haschek B, Sommerfeld-Stur I, Moestl K, Baumgartner W: Characterization of virulence factors in Escherichia coli isolated from diarrheic and healthy calves in Austria shedding various enteropathogenic agents. Vet Med 2009, 54:1–11

33-Welinder-Olsson C, Badenfors M, Cheasty T, Kjellin E, Kaijser B (2002) Genetic profiling of enterohemorrhagic Escherichia coli strains in relation to clonality and clinical signs of infection. J Clin Microbiol. 40:959-964. 10-

Peter K Fagan, Michael A Hornitzky, Karl A Bettelheim, Steven P Djordjevic (1999) Detection of Shiga-Like Toxin (stx1 andstx2), Intimin (eaeA), and Enterohemorrhagic Escherichia coli (EHEC) Hemolysin (EHEC hlyA) Genes in Animal Feces by Multiplex PC.

34.Secretion of Alpha-Hemolysin by Escherichia coli Disrupts Tight Junctions in Ulcerative Colitis Patients Hengameh Chloé Mirsepasi-Lauridsen, PhD1,2,6, Zhengyu Du, MSc3,6, Carsten Struve, PhD1 , Godefroid Charbon, PhD2 , Jurgen Karczewski, PhD3 , Karen Angeliki Krogfelt, PhD1 , Andreas Munk Petersen, PhD1,4,5 and Jerry M Wells, PhD3. Clinical and Translational Gastroenterology (2016) 7, e149; doi:10.1038/ctg.2016.3

35. Peter K Fagan, Michael A Hornitzky, Karl A Bettelheim, Steven P Djordjevic (1999) Detection of Shiga-Like Toxin (stx1 andstx2), Intimin (eaeA), and Enterohemorrhagic Escherichia coli (EHEC) Hemolysin (EHEC hlyA) Genes in Animal Feces by Multiplex PC.

36.Marques L R, Abe C M, Griffin P M and Gomes T A (1995) Association between alpha-hemolysin production and HeLa cell-detaching activity in fecal isolates of Escherichia coli. J Clin Microbiol. 33: 2707–2709.

37. Dunn JR, Keen JE, Thompson RA. Shiga-toxigenic Escherichia coli O157:H7 in Louisiana Dairy Herds. Proceedings of the 10th International Symposium on Veterinary Epidemiology and Economics. 2003: Available at http:// www.sciquest.org.nz.

38.Rahman H, Deka M. Detection and Characterization of Necrotoxin Producing Escherichia coli (NTEC) from Calves with Diarrhoea, J. Vet. Pub. Hlth. 2012; 10 (2): 97-102.

39. Schmidt H, Geitz C, Tarr PI, Frosch M, Karch H. NonO157:H7 pathogenic Shiga toxin-producing Escherichia coli: phenotypic and genetic profiling of virulence traits and evidence for locality. J Infect Dis 1999; 179: 115-123.

40.Dozois CM, Clément S, Desautels C, Oswald E, Fairbrother JM. Expression of P, S, and F1C adhesins by cytotoxic necrotizing factor 1-producing *Escherichia coli* from septicemic and diarrheic pigs. FEMSMicrobiol. Lett. 1997; 152:307-312.

41. Bertin Y, Girardeau JP, Darfeuille-Michaud A, Martin C. Epidemiological Study of pap Genes among Diarrheagenic or Septicemic Escherichia coli Strains Producing CS31A and F17 Adhesins and Characterization of Pap31A Fimbriae. J Clin Microbiol 2000, 38(4):1502-1509.

42. Dezfulian H, Batisson I, Fairbrother JM, Lau PC, Nassar A, Szatmari G, Harel J. Presence and Characterization of Extraintestinal Pathogenic *Escherichia coli* Virulence Genes in F165-Positive *E. coli* Strains Isolated from Diseased Calves and Pigs. J Clin Microbiol. 2003, 41(4):1375–1385

43. Bertin Y, Martin C, Girardeau JP, Pohl P,Contrepois M. Association of genes encoding P fimbriae, CS31A antigen and EAST 1 toxinamong CNF1-producing *Escherichia coli* strains from cattle with septicemia and diarrhea. FEMS Microbiol. Lett. 1998. 162:235–239.

44. Mainil JG, Jacquemin E, Herault F, Oswald E. Presence of*pap*-, *sfa*-, and *afa*-related sequences in necrotoxigenic *Escherichia coli* isolates from cattle: evidence for new variants of the AFA family. Can. J. Vet.Res. 1997 61:193–199.

45.Guler L, Gunduz K, Ok Ü.2008 Virulence factors and antimicrobial susceptibility of Escherichia coli isolated from calves in Turkey. Zoonoses and Public Health, v.55, p.249-257.

46. Sharaf EF, Shabana II. Prevalence and molecular characterization of Shiga toxin-producing *Escherichia coli* isolates from human and sheep in Al-Madinah Al-Munawarah. Infectio 2016. http:// dx. doi. org/10.22354/in.v21i2.651

47. Schmidt H, Karch H. Enterohemolytic phenotypes and genotypes of Shiga-toxin-producing *Escherichia coli* O111 strains from patients with diarrhea and hemolytic-uremic syndrome. J Clin Microbiol. 1996;34:2364-7.

48. Shebib ZA, Abdul Ghani ZG, Mahdi LK. First report of *Escherichia coli* O157 among Iraqi children. East Mediterr Health J. 2003;9:159---66.)

49. Shaw DJ, Jenkins C, Pearce MC, Cheasty T, Gunn GJ, Dougan G, et al. Shedding patterns of verocytotoxinproducing *Escherichia coli* strains in a cohort of calves and their dams on a Scottish beef farm. *Appl Environ Microbiol* 2004; 70: 7456-765

50.Cobbold R, Desmarchelier P. Horizontal transmission of Shiga toxin-producing *Escherichia coli* within groups of dairy calves. *Appl Environ Microbiol* 2002; 68:4148-4152.