Molecular Detection of The Virulence Genes in *Escherichia coli* Isolated From Healthy and Diarrheic Calves in southern Iraq

ABSTRACT

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

Materials and Methods: The interested genes were isolated previously from rectal stool samples obtained from 150 diarrheic and non-diarrheic calves aged one day up to six months during the period of 2016 to 2017. Rectal swabs were aseptically and randomly collected from several herds from different regions in the province of Basrah.

Results of Research: The polymerase chain reaction results showed that pathogenic *E. coli* was detected in a total of 34 out of 41 (82.9%) among diarrheagenic and non-diarrheagenic calves and distributed as 23 out of 26 (88.5%) and 11 out of 15 (73.3%) in diarrheagenic and non-diarrheagenic calves respectively. The results also showed that Stx1, hlyA and Stx2 genes showed higher incidence of distribution in both diarrheagenic and non-diarrheagenic calves in a percentage rate (69.6, 65.2,56.5) and (63.6%, 63.6%, 45.5%) respectively. Moreover, 8 different virulence gene profiles were established in the present study. Most of the isolates analyzed have at least two or three genes arrangements, and only four isolates show combination of four genes Stx1,Stx2, hlyA and afaI.

Conclusion: It is concluded that *E. coli* isolates from healthy and diarrheic calves carried various virulence genes, of which the most frequent were Stx1, Stx2, hlyA and are present in a higher percentage of isolates from both diarrheic and non-diarrheic calves.

Key words:
Escherichia coli ,Calve diarrhea, Collibacillosis

1. INTRODUCTION

In veterinary medicine, diarrhea is considered one of the most popular and vital diseases that causes huge economic losses in the neonatal animals. The diarrheal can be caused by bacteria, virus, parasites and other etiological agents while *Escherichia coli* bacteria is getting recognized as the leading cause of the diarrheal. 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 illness and diseases (1).*E.coli* also causes septicemia and diarrhea in a wide range of hosts, including man, avian and animals such as cattle, piglet, goat, foals, lambs and buffalo as well as children in developing countries (2).The diarrheagenic form of (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 usually known as Colibacillosis. Colibacillosis has been linked to clinical symptoms, such as diarrhea, dehydration, fatigue, fever, malaise, and depression. Calves between 3-5 days old could 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, Shiga toxin-producing *E.coli* (STEC) strains are also well-recognized as causes 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).

The pathogenic *E.coli* can be categorized based on serogroups, pathogenicity mechanisms, clinical symptoms, or virulence factors genes. The pathogenicity of *E. coli* based on virulence factors are occurred 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 Basrah 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 Basrah through the detection of a wide-ranging of 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: 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 aged from one day up to six months during the period of 2016 to 2017. Rectal swabs were aseptically and randomly collected from several herds which were found in different regions in the province of Basrah. The samples were collected in sterile screw capped containers and labeled to show its serial number, as well as time and date of collection. Then, the samples were transported to the laboratory of Microbiology at the College of Veterinary Medicine in ice cooled containers for immediate analysis. The *E. coli* was isolated and identified on the basis of standard technique (12).All stool samples were inoculated into tubes of freshly prepared brain heart infusion broth and incubated aerobically at 37^{o}C overnight, followed by subculturing onto 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 agar and incubated at 37°C for 24 to 48 hours. A colony with a metallic

sheen appearance were picked for further analysis using the bacteriological and biochemical tests and the API 20E system (bioMerieux, Marcy l'Etoile, France). The bacteriological and biochemical tests were included: Gram stain, culturing on (Triple Sugar Iron, Oxidase test, Catalase test, Indole producing and Urease test) were carried out for the further identification of *E.coli*, according to the previous methods of (12)**.** Following purification and sub-culturing on brain heart infusion 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 brain heart infusion agar. The tubes were vortexed and incubated at 100 ºC for 20 min. Then, 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 min. The supernatant which contain the genomic DNA was transferred into new eppendorf tubes to be 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 master mix (Bioneer, Korea) and a specific set of oligonucleotide primer (Bioneer, Korea) which is illustrated in Table 1. The PCR reaction consisted of :5µl master mix, 1µl Forward Primer (Bioneer, Korea), 1µl Reverse Primer (Bioneer, Korea), 5µl DNA Templates and the final volumes of reaction were adjusted to 20 µl by adding 8 µl of nuclease free water. The PCR amplification process was accomplished by using Thermocycler type (*Esco*, Singapore) and with cycling conditions consisting of initial denaturation at 94 ◦C for 2 min, followed by 35 cycles of amplification (denaturation at 94 ◦C for

30 sec, annealing for 30 sec, extension at 72 ◦C for 1 sec and final elongation at 72 ◦C for 7 min). The analysis of the PCR products was performed in 2% agarose gel electrophoresis. A 100bp DNA ladder (Bioneer, Korea) was used as a molecular weight marker and gels were stained with ethidium bromide and examined and photographed under 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 672bp, was verified as positive for stx1, stx2, hly, cnf2, sfa/foc, afaI, papC and afaC genes respectively.

Table1: Shows the list of primers, annealing temperatures and predicted sizes used for the detection of virulence genes of *E. coli* isolated

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

3. RESULTS AND DISCUSSION

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

Results in table 2 show the bacterial isolates from 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). Samples were cultured on specific and selective media for isolation of *E. coli*

and subjected to biochemical tests and confirm the results by Api 20 system. Gram negative rods were detected by Gram staining and the isolates produced colonies with a black metallic sheen on EMB agar and a pink appearance on MacConkey agar. So on the bases of the bacteriological, biochemical and Api20 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 found to be positive for at least one of the virulence genes as revealed in table 2. The results of conventional PCR analysis were shown in figures (a,b,c,d,e,f,g and h).

On the other hand, Table 3 reveals the prevalence of virulence genes distribution among diarrheagenic and non-diarrheagenic calves. In this table, the Stx1, hlyA and Stx2 genes showed higher incidence of distribution in both diarrheagenic and non-diarrheagenic calves in a percentage rate (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, which shows 8 different virulence profiles were established in the present study. Most of the isolates analyzed have at least two or three genes arrangements, and only four isolates show combination of four genes with profile of Stx1,Stx2, hlyA and afaI.

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

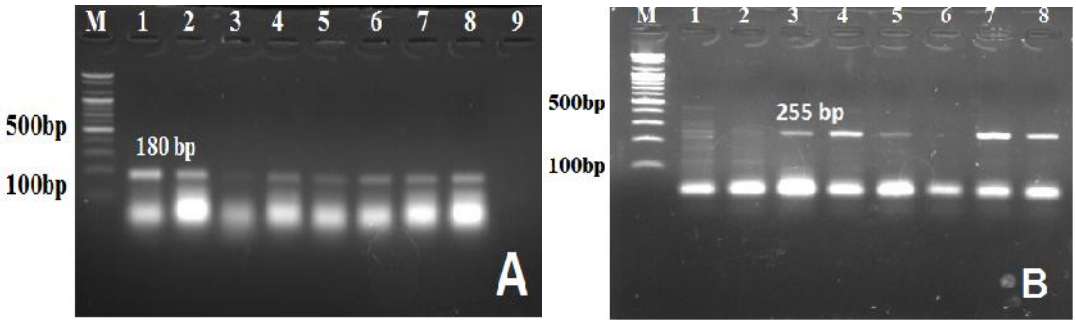
Type of sample	*E.coli* Isolation based on API 20 E system		*E.coli* Isolation based on PCR results	
	E.coli Isolation no.	*E.coli* isolation(%)	*E.coli* Isolation no.	*E.coli* isolation(%)
Diarrheagenic	26/75	34.7	23/26	88.5%
non-diarrheagenic	15/75	20	11/15	73.3
Total	41/150	27.3	34/41	82.9

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

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

Table 4: Virulence genes profiles present in *E. coli* strains isolated from diarrhea of 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



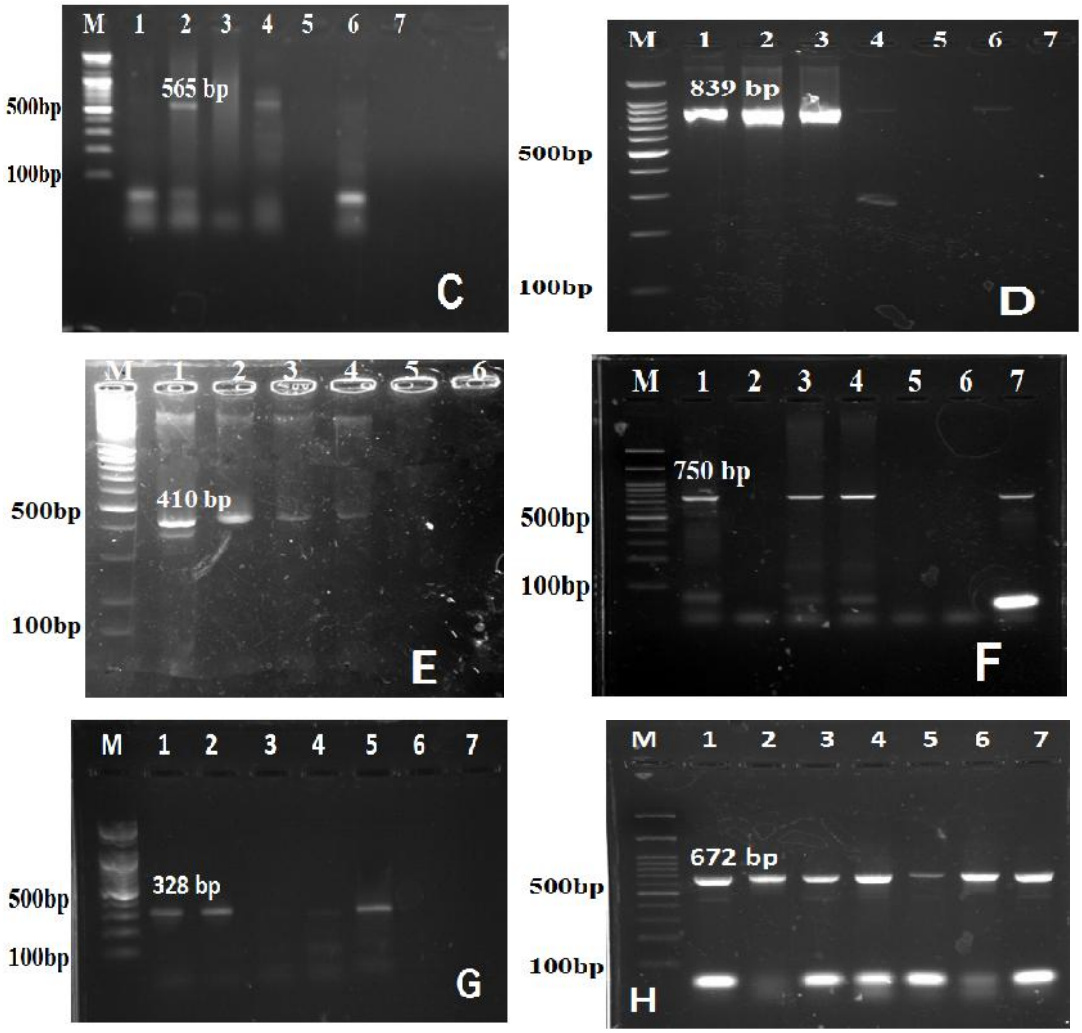


Figure 1. Agarose gel electrophoresis reveals the PCR products of : (A) Lane M: 100 bp DNA Ladder. Lanes 1- 8: PCR Products of 180 bp stx1 gene. (B) Lane M: 100 bp DNA Ladder. Lanes 3,4,5,7 and 8: PCR Products of 255 bp stx2 gene. (C) Lane M: 100 bp DNA Ladder. Lanes (2 and 4) PCR Products of 565 bp 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.

The economy of many developing countries is dependent on the production of livestock animals, which also continues to make important contributions to the local food supply. In recent years, farm animals were increasingly raised and spread particularly in southern Iraq. As a result, several livestock diseases arised. Diarrhea, caused by *E. coli*, in calves is one of these diseases, which often appears as outbreak and still one of the most common causes of death in

newly borne calves (20, 21, 22, 23). Additionally, the findings of the current study are similar to previous data that found *E. coli* was the most common bacteria isolated from fecal samples of diarrheic and healthy calves (24). Besides, the presence of pathogenic *E. coli* strains in diarrheic animals is of greater importance 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 20 E system and PCR. On the bases of the API 20 E system, the prevalence of *E. coli* infection in examined calves was 27.3%, distributed as 34.7 % in calves with diarrhea and 20 % in non-diarrheagenic calves. On the other hand, the PCR technique was 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 examined calves were 82.9 (88.5% in calves with diarrhea and 73.3% in non-diarrheagenic calves).Moreover in the current study, 7 *E. coli* isolates that were isolated from diarrheic and non-diarrheic samples they have no virulence factors. The possible explanation for this finding is the fact that maybe these strains were non-pathogenic *E. coli* and the animals have diarrhea caused by some other infectious agent (28).

On the bases of 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 encode toxic factors (stx1, stx2, hlyA and cnf) and the other four encode adhesion factors (sfa/foc, afaI, papC and afaC). The prevalence of toxic virulence gene were 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 are 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 STEC isolates was higher: 52.2% (47/90) , 33.3% (30/90) and 32.2% (29/90) respectively. In the same way, (30,31) observed 46.5% (20/43) and 55% (213/384) of the STEC isolates carried stx1 while (32) revealed that 17% of all diarrheic and healthy calves of Australian herds were infected by *E. coli* and 15.2% of *E. coli* strains harbored the shiga toxin genes including stx1, stx2 and ehly and eae genes.

Alternatively, current results were also consistent with the results of (33) and (34) where their *E.coli* isolates was PCR-positive for the hlyA Hemolysin gene. Similarly, a high occurrence of hlyA gene with *stx1* and/ or *stx2* were also detected in the study of (35). The possible explanation of occurrence a particular gene combination is the fact that particular gene is carried by plasmid and it's have ability to transferred among related bacteria(35)..Also, in the current study, the prevalence of cnf gene was low and this finding was agreed with previously studies (36).According to (36) the distribution of cnf gene in *E.coli* isolates from diarrheic and healthy animals and humans were also detected but rare and the occurrence of the cnf gene is no clear as this gene contributed more to uropathogenic *Escherichia coli* (UPEC) outbreaks in humans. So the most accepted hypothesis to existence of cnf gene which is more related to NTEC (necrotoxic *E.coli*) strains is that it occur as extraintestinal infection from UPEC and also confirmed the importance of cnf gene isolated from diarrheic and healthy animals as a potential threat to human health (29).

On the other hand, four fimbria adhesion genes were also detected ,the finding of these fimbriae factors in diarrhea condition is rare but indicators to extraintestinal pathogenic *E.coli* infection (37,38). Moreover (39,40) they finding the association between the high prevalence of fimbriae genes detection with strains that produced cytotoxic necrotizing factor that isolated from calves with septicemia or diarrhea. The current study has also revealed 8

different combinations of the virulence-associated genes in each *E.coli* isolate. Most of these isolates have at least two or three genes arrangements, and only four isolates show combination of four genes with profile of Stx1,Stx2, hlyA, afaI.

Although many researchers attempted to correlate the existence of recognized virulence profile factors with disease or severity of disease and concluded that no single factor is responsible for the virulence of STEC (41, 42,43). These variations of the gene virulence and severity may contributed to over-use and mis-use of antibiotics in intensive animal production as well as to the crowded and stressful conditions of factory farms moreover the geographical differences and climate condition also play an important role and influence on the shedding of STEC in calves (44,45).

4. CONCLUSION

It is concluded that *E. coli* isolates from healthy and diarrheic calves carried various virulence genes, of which the most frequent were Stx1, Stx2, hlyA and are present in a higher percentage of isolates from both diarrheic and non-diarrheic calves. This study also a proves that PCR is accurate, rapid and is able to isolate pathogenic *E. coli* strains obtained from a random sampling of animals.

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, Nahar1 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.

Hohmann EL (2001) Nontyphoidal salmonellosis. Clin Infect Dis 32: 263-269

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, Sperandio 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. 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.

34.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.

35. 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.

36.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.

37. 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.

38. 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

39. 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.

40. 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.

41. 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

42. 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.

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

44. 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

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