Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

Phenotypic and genetic identification of Shiga toxigenic *Escherichia coli* causing calves diarrhoea

Alaeldin Hussein Mustapha[1] ,Wafaa F. Omara[2*], Hanaa F. Salama[3]

1Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, University of Sadat City, Egypt.

2 Microbiology Department, Shebin El-Kom Lab. Animal Health Research Institute (AHRI), Agriculture Research Centre (ARC), Egypt.

3 Food hygiene Department. Damanhur Lab. Animal Health Research Institute (AHRI), Agriculture Research Centre (ARC), Egypt.

ARTICLE INFO

Keywords

Calf diarrhoea,

Shiga toxin

Escherichia coli

(STEC), VITEK

Received 04/07/2024
Accepted 06/08/2024
Available On-Line 01/10/2024

ABSTRACT

Calf diarrhea is a major cause of pre-weaning sickness in cattle operations. This study attempted to isolate *Escherchia coli (E.coli)* from diarrheic calves as well as evaluate its role by analyzing the incidence of Shiga toxin genes. The samples were examined by culturing, the VITEK test, and molecular screening of *E. coli* virulence genes. The biochemical evaluation showed that the isolates were positive for Indole, Methyl red, Nitrate reduction, Lactose, and Arabinose sugar fermentation tests, while Voges Proskauer, Citrate utilization, Urease, and H_2S tests were negative, and these results were confirmed using VITEK® 2 Compact as the isolate gave 98% similarity to the features of *E. coli* that were recognized by the standard card for Gram negative. From the 150 examined samples, 55 samples (37%) were positive for *E. coli*. Only ten *E. coli* strains tested positive for O157, according to serological analysis. The genetic examination of stx (1 and 2) genes demonstrated that three samples identified as *E. coli* O157 exhibited both stx (1 and 2) genes, one of them exhibited stx1, and two isolates carried stx2 only, whereas four *E. coli* O157 strains did not exhibit any stx genes. The total isolates of *E. coli* O157 obtained from diarrheic calves included *rfb*E and *pho*A genes. *E. coli O157* detected in 18.2% of positive *E. coli* samples is considered a major hazard, as the isolated strains contain *stx*1, *stx2*, *rfbE*, and *pho*A virulence genes. .

1. INTRODUCTION

Colibacillosis is a deadly disease that affects young calves during their initial days of life, resulting in significant mortality losses (Fathy et al., 2019). This multifactorial disease included various components such as calf immunological state, habitat, and hygiene conditions (Lorenz, 2006), with mortality rate being the most relevant risk factor (Muluken et al., 2017). *Escherichia coli* is the primary pathogenic agent, playing an essential role in illness incidence (Tenallon et al. 2010). However, only a few extremely evolved pathogenic *E. coli* strains can cause intestinal or extra intestinal disorders that result in substantial financial consequences (Sousa, 2006). *E. coli* that have enterotoxigenic genes have been detected in calves with diarrhoea (Dereje, 2012; Masud et al., 012). and the virulence factors were also discussed by Ashenafi and Tesfaye (2016), as the authors noticed that young calves and inadequate feeding were substantially linked with the isolation of *E. coli*. The O157 strain of *E. coli* is regarded as a foodborne infectious agent, causing occasional to severe outbreaks all over the world. The morbidity and mortality linked to Shiga toxin-producing *E. coli* (STEC) outbreaks have been highlighted as the organism's hazard to public health (Abotalp et al., 2017). STEC is considered the most contested and harmful strain of *E. coli*. It thrives mostly throughout cow guts (Ahmadi et al., 2020). Momtaz et al. (2012) reported that the pathogens of STEC strains are controlled by virulence factors such as the production of Stx1 and/or Stx2 Several serotypes have been related to serious human infections, including O157; a common STEC serotype. Shiga toxins (Stxs) can cause diarrhea and hemorrhagic colitis (HC), with potentially catastrophic outcomes (Nüesch-Inderbinen et al., 2018). Several studies have studied the capacity of STEC to cause infections in humans and animals (Tavakoli and Pourtaghi, 2017; Zafarane et al., 2017). Therefore, the current study attempted to extract and characterize *E. coli*, followed by the molecular identification of STEC isolated from diarrheic calves.

2. MATERIAL AND METHODS

2.1. Sampling

From September 2021 to March 2022, a study was undertaken to assess the prevalence of *E. coli* in diarrheic calves (3 months of age). The study also assessed risk factors for calf diarrhoea and the presence of STEC virulence genes so 150 faecal samples were gathered from diarrheic calves on diverse farms in Menoufia governorate, Egypt.

2.2. Isolation and identification of enterotoxigenic E. coli

MacConkey broth tubes (HIMEDIA, M1125) were supplemented with reversed Durham tubes. Inoculated tubes were incubated at 37 °C for 24 hours. One millilitre of a positive MacConkey tube was transferred into another MacConkey broth tube and incubated at 44 °C for 24 hours. Loopfuls of positive MacConkey broth tubes were streaked on Eosin Methylene Blue agar medium (EMB) (HIMEDIA, M317) and incubated at 37°C for 24 hours. The putative

* Correspondence to: wafaaomara83@gmail.com

colonies were metallic green in colour. Also On cefixime tellurite-sorbitol MacConkey medium at 37 ºC for 24 hours, the colonies appeared colorless.

2.2.1. Enrichment according to ISO 16649-2 (2001)
The suspected colonies were purified and placed in slope nutrient agar tubes (Oxoid, 75312) for further study.

2.2.2. Identification of enterotoxigenic E. coli
Tested morphologically as Gram-negative bacilli, the purified isolates were tested biochemically by sugar fermentation tests, indol, methyl red, Voges-Proskaur, citrate utilization, H2S production, urease, catalase, oxidase, and nitrate reduction tests according to Quinn et al. (2002).

2.2.2.5. Bacterial isolates characterization
It was performed with the VITEK®2 compact Following the company's instructions (Biomeriux, 2006). The turbidity was adjusted to the equivalent of 0.5–0.6 McFarland turbidity. Analysis was done using Gram-negative bacterial identification cards. Cards were automatically read every 15 minutes.

2.2.3. Serological Identification of E. coli
The isolates were serologically identified for O antigen according to Kok et al. (1996) by using rapid diagnostic *E. coli* antisera sets (DENKA SEIKEN Co., Japan) for diagnosis of the enteropathogenic types at the Animal Health Research Institute, Cairo.

2.2.4. Extraction of DNA and genetic identification.
Using the QIAamp Mini Kit (number 51304), it uses a silica membrane to purify nucleic acids from various sources. As described in Sambrook et al. (1989), ten isolates were tested for the virulence genes stx (1 and 2), phoA, and O157 rfbE using PCR (Biometra, Germany). The products were amplified at 614, 779, 720, and 259 bp, respectively (Table 1).
A multi-purpose, high-gel-strength agarose is suitable for a wide range of molecular biology techniques. Multi-agarose, with its high gel strength and exclusion limits, could effectively separate large DNA fragments with shorter running times.

Table (1) Oligonucleotide primers sequences Cycling conditions of the primers during cPCR.

Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	Final extension	No. of cycles	Extension	Annealing	Secondary denaturation	Primary denaturation	Reference
*stx*1	ACACTGGATGATCTCAGTGG		72˚C	35	72˚C	58˚C	94˚C	94˚C	Dipineto et al. 2006
	CTGAATCCCCCTCCATTATG	614	10 min.		45 sec.	40 sec.	30 sec.	5 min.	
*stx*2	CCATGACAACGGACAGCAGTT		72˚C	35	72˚C	55˚C	94˚C	94˚C	
	CCTGTCAACTGAGCAGCACTTTG	779	10 min.	35	45 sec.	40 sec.	30 sec.	5 min.	
E. coli	CGATTCTGGAAATGGCAAAAG		72˚C	35	72˚C	58˚C	94˚C	94˚C	Hu et al. 2011
*pho*A	CGTGATCAGCGGTGACTATGAC	720	10 min.		45 sec.	40 sec.	30 sec.	5 min.	
O157	CGGACATCCATGTGATATGG		72˚C	35	72˚C	58˚C	94˚C	94˚C	Paton and Paton, 1998
*rfb*E	TTGCCTATGTACAGCTAATCC	259	10 min.		45 sec.	40 sec.	30 sec.	5 min.	

3. RESULTS

The colonies appeared as a green metallic sheen on EMB agar (Figure 1A) after 24 hours of incubation at 37 °C. On cefixime tellurite-sorbitol MacConkey medium, the colonies appeared colorless (non-sorbitol fermenting), indicating that it may be *E. coli* O157 figure (1B). The biochemical evaluation of the isolated *E. coli* revealed positive Indole, Methyl Red, Nitrate Reduction Tests, Lactose, and Arabinose Sugar Fermentation. While Voges Proskauer, Citrate utilization, Urease and H2S tests were negative (table 2). These results were confirmed using VITEK® 2 compact, which resulted in 98 % similarity to the biochemical features of *E. coli*, and fulfilled the identification criteria of the standard card for gram-negative, as shown in Table 3. While the serological screening of the isolates revealed that out of the 55 *E. coli* isolates, only 10 were identified as *E. coli* O157 (Fig. 3). Furthermore, in Fig. 4, the molecular screening of s*tx* 1 and *stx* 2 genes at 614 and 779 bp, respectively, revealed that 3 of *E. coli* O157 carried both *stx* genes, one isolate carried stx1 only and two isolates carried *stx2* only while 4 of *E. coli* O157 were negative for both stx genes. Also, Figures 5 and 6 showed that all *E. coli* O157 isolates had the rfbE and phoA genes, which were found to be 259 bp and 720 bp, respectively.

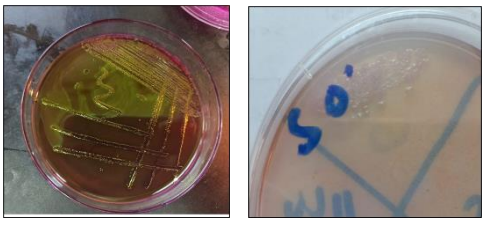

Figure (1A) green metallic sheen of *E. coli* on EMB Figure (1B) colourless colonies of *E. coli* O157 on cefixime tellurite-sorbitol MacConkey medium

Table (2) Biochemical reactions for identification of *E. coli* isolated from diarrheic calves

Biochemical test	*E. coli*
Motility	V
Indole	+
Methyle red	+
Voges Proskuaer	-
Citrate utilization	-
Urease	-
H2S	-
Nitrate reduction	+
Gelatin liquefaction	-
ODC	V
LDC	V
Arginine dihydrolase	V
ONPG	+
Sugar fermentation	
Lactose	+
Sucrose	V
Dulcitol	V
Salicin	V
Arabinose	+
Inositol	-
Xylose	V

Table (3) Biochemical details of *E. coli* isolated from diarrheic calves using VITEK® 2 compact

2	APPA	-	3	ADO	-	4	PyrA	-
5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	+	11	BNAG	-	12	AGLTp	-
13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+
20	dMNE	+	21	BXYL	-	22	BAlap	-
23	proA	+	26	LIP	-	27	PLE	-
29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	+	35	dTRE	+
36	CIT	-	37	MNT	-	39	5KG	-
40	ILATK	+	41	AGLU	-	42	SUCT	+
43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+
53	IHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	IMLTa	-
62	ELLM	-	64	ILATa	-			

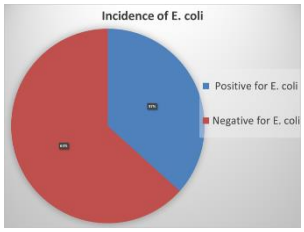

Fig. (2) The prevalence of *E. coli* isolated from diarrheic calves

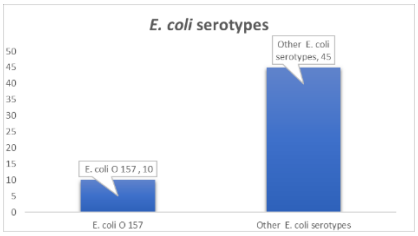

Fig. (3) The prevalence of *E. coli* O157 serotype that detected in diarrheic calves

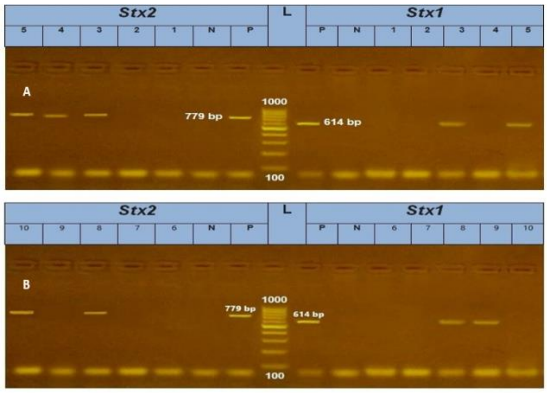

Fig. 4 (A&B) Agarose gel electrophoresis of *stx*2 (shiga toxin 2) (779bp) and *stx1* (shiga toxin 1) (614 bp) genes identified by PCR from *E. coli O*157 strains isolated from diarrheic calves. Lane L: 100 bp ladder. Lane pos.: Control positive (*E. coli*) of *stx*2 (779bp) and *stx*1 (614 bp) genes. Lane neg.: Control negative (*S. aureus*). Lanes 1, 2, 6 and 7 are negative for both *stx*2 and *stx*1. Lanes 3, 5 and 8 are positive for both *stx*2 and *stx*1 Lane 4 and 10 is neg. for stx_1 while pos. for *stx*2. Lane 9 is pos. for *stx*1 while neg. for *stx*2.

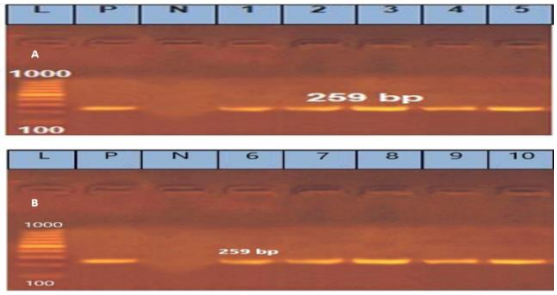

Fig. 5 (A&B) Agarose gel electrophoresis of *rfb*E (259bp) gene identified by PCR from *E. coli O157* strains isolated from diarrheic calves. Lane L: 100 bp ladder as molecular size DNA marker. Lane P: Control positive *(E. coli)* of. *rfb*E (259bp) gene. Lane N: Control negative (*S. aureus*). Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 are positive for *rfb*E (259bp) gene

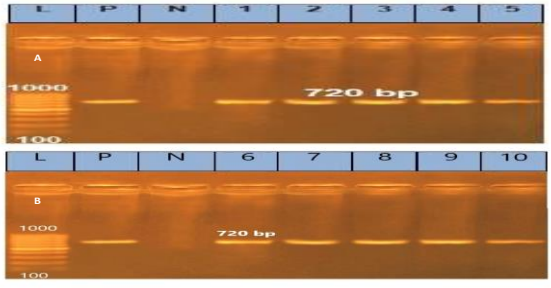

Fig. 6 (Aand B) Agarose gel electrophoresis of *pho*A *(*720bp) gene identified by PCR from *E. coli O157* strains isolated from diarrheic calves. Lane L: 100 bp ladder as molecular size DNA marker. Lane P: Control positive (*E. coli*) of *pho*A (720bp) gene. Lane N: Control negative (*S. aureus*). Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 are positive for *phoA* (720bp) gene.

4. DISCUSSION

Escherichia coli causes diarrhoea in farm animals, as its strains are classified as enterotoxigenic *E. coli* (ETEC) or STEC based on their virulence and clinical symptoms in the host (Dereje 2012). Our goal is to molecularly characterize *E. coli* isolated from neonatal diarrheic calves and look into the virulence factors linked to the diarrheic infection caused by *E. coli* in young calves. The prevalence of *E. coli* in the samples was 37%, which is similar to Gebregiorgis and Tessema (2016), who found that 36.8% of diarrheic calves were positive for *E. coli*. Mohammed et al. (2019) found a higher incidence of *E. coli* isolated from calf diarrhoea: 46.4%; Masud et al. (2012); Dereje (2012); and Paul et al. (2010) were 44%, 43.1% and 76%, respectively. These differences in *E. coli* prevalence might be related to variances in the research region, calves' ages and management, as well as hygienic techniques (Radostits et al., 2007). The incidence of O157 in the current investigation was 18.18%, in agreement with Kang et al. (2004) recovery of 15.79% of O157 from calves with diarrhoea. Furthermore, Nguyen et al. (2011) identified O157 (8.9%) from diarrheic calves. The presence of two strains of serotype O157 raises concerns about human health (Blanco et al. 2003).

Milk and other dairy products are frequently polluted with O157 by direct contact with faeces, resulting in gastrointestinal tract illness (Bélanger et al. 2011).

The molecular characterization of Shiga toxin genes *stx*1 and *stx*2 revealed the presence of these genes in the isolated *E. coli* O157. Similar studies have also targeted testing for *stx* genes, for example, *stx*2 (Lamey et al., 2013) and *stx*1 (Naser, 2016). The importance of Shiga toxins in *E. coli* pathogenicity depends on their responsibility for *the* attachment and binding of bacteria to glycolipid on the Gb3 sites on the cell surface, leading to the cessation of protein synthesis and consequently the death of bacterial cells (Kaper et al. 2004). In addition to their main role in the production of enterotoxins that exert diarrhoea as well as alter the acid-base balance of the blood and small intestine (Nagy and Fekete, 1999; Nataro and Kaper, 1998). These results were unparallel to those of Karmali et al. (2003), who reported that *stx*1 gene was not detected, while the *stx*2 gene was found to be 93.1%. Akter et al. (2016) examined 25 *E. coli* isolates and found that 7 isolates were positive for *Stx1* and none of them were *stx*2 positive, however, Wieler et al. (2007) and Abotalp et al. (2017) reported that *stx*2 gene percentages ranged between 4.5% and 43.75% in Germany and Egypt, respectively. Awad-Masalmeh, (2004) showed that the incidence of *stx1* and *stx2* was 10.1% and 17.8%, respectively. Further, the prevalence of STEC in diarrheic calves was 26.3% (41 isolates) and *stx*1 gene was the most prevalent variant among the isolates (Taghadosi et al. 2018). These genes have been widely used as specific primers and probes for the *E. coli* O157 serotype. Basli et al. (2016) used *rfb*E gene to detect *E. coli* O157:H7 by PCR. The *rfb*E gene is responsible for the production of the lipopolysaccharide (LPS) O side chain of the STEC O157: H7 cell surface (Shahzad et al., 2021). Also, the *pho*A gene encodes for alkaline phosphatase in *E. coli*. This wide range in gene frequency necessitated further investigation into the actual distribution and prevalence of this gene in various animal species, its role in interspecies transmission, and another related factor (Fathy et al. 2019).

5. CONCLUSIONS

E. coli O157, which was detected in 18.18% of positive *E. coli* samples, is considered a major hazard as the isolated strains contain *stx*1, *stx*2, *rfb*E and *pho*A virulence genes. More research should be conducted on a large number of animals to explore the microbiological origins of calf diarrhoea and preventative methods.

CONFLICT OF INTEREST

No conflict of interests.

6. REFERENCES

1. Abotalp, E.H., Abdeen, E., Moustafa, A.E.D.H. and Mohamed, S.R., 2017. Molecular Detection of Virulence Genes of *Escherichia coli* O157 Isolated from Different Sources. Alexandria Journal for Veterinary Sciences, 53,2, 38-44.
2. Ahmadi, E., Mardani, K. and Amiri, A., 2020. Molecular detection and antimicrobial resistance patterns of Shiga toxigenic *Escherichia coli* isolated from bovine subclinical mastitis milk samples in Kurdistan, Iran. Archives of Razi Institute, 75,2, 169-177.
3. Akter, M.M., Majumder, S., Nazir, K.M. and Rahman, M., 2016. Prevalence and molecular detection of shiga toxin producing *Escherichia coli* from diarrheic cattle. Journal of the Bangladesh Agricultural University, 14,1, 63-68.
4. Ashenafi, G.E. and Tesfaye, S.T., 2016. Characterization of *Escherichia coli* isolated from calf diarrhea in and around Kombolcha, South Wollo, Amhara Region, Ethiopia. Trop. Anim. Health Prod. 48, 273–281.
5. Basli, A. A. A., Mohamadein, M. M. and Farrag, R. M. 2016. Detection and virulence of *Escherichia coli* O157: H7 isolates using multiplex PCR. Wulfenia Journal, 23, 41-48.
6. Bélanger, L., Garenaux, A., Harel, J., Boulianne, M., Nadeau, E. and Dozois, C. M., 2011. *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic E. coli. FEMS Immunology & Medical Microbiology, 62, 1–10.
7. Biomeriux VITEK-2 Compact ref Manua –Ref-414532. 2006. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M7A5. Normal committee for clinical laboratory standards. Wayne, https://clsi.org/media/1928/m07ed11_sample.
8. Blanco, J., Blanco, M., Blanco, J.E., Mora, A., González, E.A., Bernárdez, M.I., Alonso, M.P., Coira, A., Rodriguez, A., Rey, J., Alonso, J.M. and Usera, M.A. 2003. Verotoxin-producing *Escherichia coli* in Spain: prevalence, serotypes, and virulence genes of O157:H7 and non-O157 VTEC in ruminants, raw beef products, and humans. Exp Biol Med ,Maywood, 228, 345-351.
9. Dereje, W., 2012. Isolation and identification of Enterobacteria species from diarrheic calves in and around Addis Ababa, Ethiopia.
10. Dipineto, L., Santaniello, A., Fontanella, M., Lagos, K., Fioretti, A. and Menna, L.F. 2006. Presence of Shiga toxin-producing *Escherichia coli* O157:H7 in living layer hens. Letters in Applied Microbiology 43, 293–295.
11. Fathy, H., Abdeen, E., Moustapha, A.E.H. and Younis, G., 2019. Development of PCR and multiplex-PCR for detection of some *Escherichia coli* virulence genes from bovine neonates Diarrhea. Journal of Current Veterinary Research, 1,1,11-16.
12. Gebregiorgis, A. and Tessema, S.A. 2016. Characterization of *Escherichia coli* isolated from calf diarrhea in and around Kombolcha, South Wollo, Amhara Region, Ethiopia Trop Anim Health Prod., 48, 273–281.
13. Hu, Q., Tu, J., Han, X., Zhu, Y., Ding, C. and Yu, S. 2011. Development of multiplex PCR assay for rapid detection of Riemerellaanatipestifer, Escherichia coli, and Salmonella enterica simultaneously from ducks. Journal of Microbiological Methods 87, 64–69.
14. ISO ,International Standards Organization 16649-2, 2001. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of glucuronidase- positive Escherichia coli- Part 2; Colony count technique at 44ºC using-bromo-4-chloro-3-indoly-D-glucuronide. International Standards Organization, Geneva, Switzerland.
15. Kang, S.J., Ryu, S.J., Chae, J.S., Eo, S.K., Woo, G.J. and Lee, J.H., 2004. Occurrence and characteristics of enterohemorrhagic *Escherichia coli* O157 in calves associated with diarrhoea. Veterinary microbiology, 98,3-4, 323-328 .
16. Kaper, J.B., Nataro, J.P. and Mobley, H.L., 2004. Pathogenic escherichia coli. Nature reviews microbiology, 2,2, 123-140.
17. Kok, T., Worswich, D. and Gowans, E. 1996. Some serological techniques for microbial and viral infections. In Practical Medical Microbiology ,Collee, J.; Fraser, A.; Marmion, B. and Simmons, A., eds., 14th ed., Edinburgh, Churchill Livingstone, UK.
18. Lamey, A., Ammar, A.M., Zaki, E., Khairy, N., Moshref, B.S., Refai, M.K., 2013. Virulence factors of *Escherichia coli* isolated from recurrent cases of clinical and subclinical mastitis in buffaloes. Int. J., 4, 86-94.
19. Lorenz, I. 2006. Diarrhoea of the young calf: an update. Proceedingsof the XXIV World Buiatrics Congress. 130-8.
20. Masud, M., Fakhruzzaman, M. and Nazir, H., 2012. Isolation of *E. coli* from apparently healthy and diarrheic calves in Bangladesh and their antibiogram. J. Bang Soc. Agric. Sci. Technol. 9, 45–48.
21. Mohammed, S.A.E.M., Marouf, S.A.E.M., Erfana, A.M., El, J.K.A.E.H., Hessain, A.M., Dawoud, T.M., Kabli, S.A. and Moussa, I.M., 2019. Risk factors associated with *E. coli* causing neonatal calf diarrhea. Saudi journal of biological sciences, 26,5, pp.1084-1088.
22. Momtaz, H., Safarpoor, D., Taktaz, T., Rezvani, A., Yarali, S., 2012. Shiga toxin-producing *Escherichia coli* isolated from bovine mastitic milk: serogroups, virulence factors, and antibiotic resistance properties. Sci World J., 618709 .
23. Muluken, T., Niguse, A., Mu-uz, G., Nirage, H., Wassie, B., Gashaw, B. and Birhanu, T., 2017. Major causes and risk factors associated with calf mortality in small scale dairy farms in gondar town. Ethiopia Acad. J. Anim. Dis. 6 ,3, 67–74.
24. Naser, H.H., 2016. Detection of shiga toxin producing *Escherichia coli* isolated from mastitis milk of sheep by qPCR technique. Al-Qadisiyah Journal of Veterinary Medicine Sciences.
25. Nguyen, T.D., Vo, T.T. and Vu-Khac, H., 2011. Virulence factors in *Escherichia coli* isolated from calves with diarrhea in Vietnam. Journal of Veterinary Science, 12,2, 159-164.
26. Nüesch-Inderbinen, M., Morach, M., Cernela, N., Althaus, D., Jost, M., Mäusezahl, M., Bloomberg, G. and Stephan, R., 2018. Serotypes and virulence profiles of Shiga toxin-producing *Escherichia coli* strains isolated during 2017 from human infections in Switzerland. International Journal of Medical Microbiology, 308,7, 933-939 .
27. Paul, K., Khan, M. and Mahmud, S., 2010. Isolation and characterization of *E. coli* from buffalo calves in some selected areas of Bangladesh. Bangl. J. Vet. Med. 8, 23–26.
28. Paton, A.W. and Paton, J.C. 1998. 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, 36,2: 598–602.
29. Quinn, P.J., Markey, B.K., Carter, M.E., Donnelly, W. J. C., Leonard, F.C., Maguire, D., 2002. Veterinary Microbiology and Microbial Disease, 2nd Edition. Wiley Blackwell .
30. Radostits, O. M., Gay, C. C., Hinchcliff, K. W. and Constable, P. D. ,Eds. 2007. Veterinary Medicine, 10th Edition, 847-888, Saunders, Philadelphia.
31. Sambrook, J., Fritscgh, E.F. and Mentiates, 1989. Molecular coloning. A laboratory manual.Vol ., Cold spring Harbor Laboratotry press, New York.

32. Shahzad, A., Ullah, F., Irshad, H., Ahmed, S., Shakeela, Q. and Mian, A.H., 2021. Molecular detection of Shiga toxin-producing *Escherichia coli* ,STEC, O157 in sheep, goats, cows and buffaloes. Molecular biology reports, 48,8,, 6113-6121.
33. Sousa, C.P. 2006. *Escherichia coli* as a specialized bacterial pathogen. RevBiolCiên Terra. 6:341-52.
34. Taghadosi, R., Shakibaie, M.R., Alizade, H., Hosseini-Nave, H., Askari, A., Ghanbarpour, R., 2018. Serogroups, subtypes and virulence factors of shiga toxin-producing *Escherichia coli* isolated from human, calves and goats in Kerman, Iran. Gastroenterol. Hepatol. Bed. Bench 11 ,1,, 60–67.
35. Tavakoli, M. and Pourtaghi, H., 2017. Molecular detection of virulence genes and multi-drug resistance patterns in *Escherichia coli* ,STEC, in clinical bovine mastitis: Alborz province, Iran. Iran J Vet Res 18, 208-211 .
36. Zafarane, S., Houri, H., Kazemian ,H., Heidari, H., Amiri, P. and Tabarraei, B., 2017. Characterization of virulence genes, serogroups and antimicrobial susceptibility of Shiga toxin producing *Escherichia coli* isolated from bovine mastitic milk in Tehran, Iran. Tropl Biomed 34, 295-304.