Bangl. J. Vet. Med. (2015). 13 (1): 45-51 ISSN: 1729-7893 (Print), 2308-0922 (Online)

INVESTIGATION OF PATHOGENIC *ESCHERICHIA COLI* FROM DIARRHEIC CALVES IN SELECTIVE AREA OF BANGLADESH

A. K. M. A. Islam[1], M. Rahman[2], A. Nahar[1], A. Khair[1] and M. M. Alam[1*]

[1]Department of Medicine, [2]Department of Microbiology and Hygiene, Faculty of Veterinary Science
Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

ABSTRACT

Molecular technique was used to investigate the prevalence of virulent diarrheic genes in pathogenic *Escherichia coli* and their antibiotic sensitivity patterns. A hundred samples from 100 different diarrheic calves from mid-north-western part of Bangladesh were screened for the presence of virulence factors associated with diarrhea. Following isolation and identification on the basis of cultural, morphological and biochemical properties, the presence of the virulence genes such as *eaeA, bfpA, elt, est, stx1 and stx2* were examined using PCR. Antimicrobial susceptibility of 57 *E. coli* was determined by agar disk diffusion method for 8 antimicrobial agents. Out of 100 samples 57 (57%) were found to be positive for *E. coli* and their distribution rates according to their age, breed and sex were 66.7% (6 days old), 85.7% (Sahiwal breed) and in 64.2 % (female calves) respectively. Among 57 *E. coli* isolates, only 16 isolates were analyzed for the detection of the said genes. Among them, only *eaeA* gene was detected in 2 *E. coli* isolates (12.5 %). Antibiotic resistance patterns revealed that Oxacillin, Rifampicin and Penicillin were 100% resistant followed by Erythromycin which was more than 80% resistant. In case of Amoxicillin and Tetracycline, about 59.65% and 61.40% were found to be resistant respectively whereas all 57 *E. coli* isolates showed moderately susceptible (30%) to Cefuroxime, a second generation Cephalosporin. Therefore, none of the eight antimicrobials studied can not be recommended as single best therapeutic agent for the treatment of neonatal calf diarrhea. In addition, this study indicated that diarrhea in calves in these locations can be ascribed to mainly *Enteropathogenic E. coli* (*EPEC*) which was atypical (only contained the *eaeA* genes but not *bfpA*). However, further studies are necessary to characterize the isolated *eaeA* gene positive *E. coli* by serotyping, tissue culture assay and other molecular techniques to find out the potentiality of those virulent genes contributing pathogenicity of *E. coli* causing diarrhea in calves.

Key words: Prevalence; polymerase chain reaction; virulence genes; antibiotic sensitivity; atypical *EPEC*

INTRODUCTION

Infectious diseases, especially diarrhoea, are among the most important disorders in calves (Sivula *et al*., 1996; Bendali *et al*., 1999a; Svensson *et al*., 2003). Diarrhoea in young calves is a syndrome of great aetiological complexity that causes economic losses directly through mortality and indirectly from poor growth. In addition to the influence of various environmental, managemental, nutritional and physiological factors, the infectious agents capable of causing diarrhoea in the neonatal calf are numerous.

Bacterial infections are an important cause of morbidity and mortality in large animal neonates (Fecteau *et al*., 1997). In addition to economic losses, diarrhoea in livestock is very important because of the public health implications. Numerous infectious agents causing diarrhoea in animals are zoonotic and have been associated with food-borne diseases (Trevejo *et al*., 2005). The diarrheal cause may be bacteria, virus, parasites and other etiological agents while *E.coli* is getting recognized as leading cause. *E. coli* produces septicaemia and diarrhoea in a wide range of hosts including man, avian and animals such as cattle, piglet, goat lings, foals, lambs and buffalo. Calves are most vulnerable to *E. coli* infection where age group appears to be of mostly 1-3 days of age. The pathogenicity of *E. coli* is associated with a number of virulence factors, including Shiga toxin1 (encoded by the *stx1* gene), Shiga toxin 2 (encoded by the *stx2* gene), intimin (encoded by the *eaeA* gene), bundle forming pilus (encoded by *bfp* gene), and enterohaemolysin (encoded by the *Ehly* gene) (Kang *et al*., 2004).The strains inducing gastroenteric disease are known as Diarrhegenic *E. coli* (*DEC*). *DEC* are subdivided in different pathotypes based on their virulence properties (Nataro *et al*., 1998). Enteropathogenic *E. coli* (*EPEC*), Enterotoxigenic *E. coli* (*ETEC*), Enterohemorrhagic (*EHEC*), Enteroinvasive *E. coli* (*EIEC*), Enteroaggregative *E. coli* (*EAggEC*), and Diffusely Adherent *E. coli* (*DAEC*).

Enteropathogenic *Escherichia coli* (*EPEC*) are defined as diarrheagenic *E. coli* that produce a characteristic histopathology known as attaching and effacing (A/E) on intestinal cells through encoding intimin, but that do not produce Shiga, Shiga-like or verocytotoxins.

*Corresponding e-mail address: asamahbub2003@yahoo.com

These are major causes of diarrhea among children & neonatal animals in developing countries (Carneiro *et al*., 2006). The primary virulence factor in *EPEC* is the *eae* gene that encodes intimin, located in the locus of enterocyte effacement (LEE). The LEE genes facilitate intimate adherence to host cells and the formation of the characteristic attaching and effacing (A/E) lesions (Bolton, 2011). Human *EPEC* also carry a plasmid called the EPEC adherence plasmid (*pEAF*) which includes the *bfp* gene, encoding the bundle-forming pili required for adherence and clustering on cultured epithelial cells (Orden *et al*., 2002; Hornitzky *et al*., 2005). However, not all *EPEC* are *pEAF* positive, and many nonhuman isolates lack this plasmid. The former (*pEAF* positive) are referred to as typical (*tEPEC*) and the latter as atypical EPEC (*aEPEC*). Typical *EPEC* are transmitted from human to human via the faecal-oral route, and as countries become more industrialized, the relative incidence of *tEPEC* infection decreases, probably as a result of improved sanitation. However, the epidemiology of *aEPEC* is different; associated diarrhoeal disease remains a public health issue even in developed countries (Blanco *et al*., 2006). Although a strong association between *aEPEC* and endemic diarrhoea has not been demonstrated, large outbreaks have been reported (Moller-Stray *et al*., 2012). Sporadic cases or large *STEC* outbreaks in humans are associated with the consumption of raw or undercooked meat of food animals and other foods contaminated by animal faeces, and also by contact with *STEC*-positive animals or with their environment (Paton and Paton, 1998b). Furthermore, the emergence of *aEPEC* in Europe, the United States of America and other industrialized countries is a cause for concern as *aEPEC* more readily acquire the bacteriophage-mediated verocytotoxin genes, thus developing the ability to cause more serious illness including enterohaemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Trabulsi *et al*., 2002). Despite this , *aEPEC* are not as well characterized as *tEPEC*, and research is now required to address this deficit (Dulguer *et al*., 2003; Blanco *et al*., 2006; Abe *et al*., 2009). Numerous studies in several countries have shown that cattle are implicated as the principal reservoir of *STEC* in their gastrointestinal tract, the organism has also been reported in sheep, goats, water buffalos, and deer (Wieler *et al*., 1998; Osek *et al*., 2000). However, the organism did not appear to be pathogenic in older calves and adults (Kang *et al*., 2004).

Antibiotic resistance to bacteria is a serious and growing phenomenon and has emerged as one of the pre-eminent public health concerns of the 21st century. In Bangladesh complete understanding on the occurrence of antimicrobial resistance in *E.coli* is largely unknown. The choice of which antibiotic is likely to be most effective requires knowledge of potential resistance. The practice of under dosing, over dosing as well as indiscriminate usage of drugs are not uncommon in Bangladesh. As a result, bacterial strains are being developed which are multidrug resistant and new types of antibiotics are required for the prevention and control of diseases. Considering the above questions this study was aimed to investigate the presence of *E. coli* causing diarrhea in calves and to detect their virulence factors as well as the study of their antibiotic sensitivity patterns.

MATERIALS AND METHODS

The study was conducted from January 2014 to June 2014 on the calves of Bathan regions in Sirajgonj district. Diarrheic samples from this region were brought to the Laboratory of Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh as soon as possible after collection for further examinations. Bathans are considered the most important production zone of dairy cattle in Bangladesh where calves are reared in clusters.

.

Collection of samples

A hundred diarrheic faecal samples were collected from the Sirajgonj district of Bangladesh. Each sample was aseptically collected in a sterile stool pot and transported to the laboratory of department of Medicine as soon as possible for further bacteriological examinations. Samples were processed within 24–48 h after reception.

Isolation of the bacteria

For the isolation and identification of *E. coli* standard methods as described by Cowan, 1985 were followed. Nutrient broth (NB) was used for primary culturing of *E. coli* organisms that were present in the collected faecal samples. For the differentiation of the bacteria MacConkey (MC) agar medium was used followed by Eosin Methylene blue (EMB) agar medium which was used as a selective medium and these are used according to the methods as described by Cheesbrough, 1984.

Identification and characterization of the bacteria

The isolated bacteria was confirmed with their distinctive cultural characteristics, morphology with Gram's staining and biochemical tests according to Cowan (1985) and Cheesbrough (1984).

Detection of virulent genes by PCR

Among 57 *E. coli* isolates, only 16 *E. coli* isolates were subjected to PCR for detection of six diarrheic pathogenic genes **(***eaeA, bfpA, stx1, stx2, est and elt).*

Bacterial DNA extraction was done through boiling methods. For the extraction of genomic DNA of *E. coli,* a single colony of *E. coli* was taken in 1000 µl of TE buffer in Eppendorf tube. The mixture was then vortexed and boiled at 100°C for 10 minutes. After boiling the tubes were immediately placed on ice for 5 minutes followed by centrifugation at 14,000 rpm for 5 minutes. The supernatant was collected and stored at -20°C which was used as template DNA. The base sequences, PCR conditions and predicted sizes of the amplified products for the specific oligonucleotide primers used in the study were shown in the Table 1.

Analysis of the PCR products was then carried out by agar gel electrophoresis method at 50 Volt for 60 minutes using 1% agarose gel stained with ethidium bromide. Finally the PCR products were visualized under UV transluminator.

Table 1. The primers used in PCR for detection of 6 pathogenic genes in *E. coli*

Primer name	Sequence from 5' to 3'	Amplicon size (bp)	Target Gene	References
EAE 1	AAACAGGTGAAACTGTTGCC	454	*eaeA*	Yuluo *et al*., 2010
EAE 2	CTCTGCAGATTAACCTCTGC			
BfpA-f	AATGGTGCTTGCTTGCGGCTTGCTGC	324	*bfpA*	Hinenoya *et al.*, 2009
BfpA-r	GCCGCTTTTATCCAACCTGGTA			
EVT1	CAACACTGGATGATCTCAG	349	*stx1*	Yuluo *et al*., 2010
EVT2	CCCCCTCAACTGCTAATA			
EVS-1	ATCAGTCGTCACTCACTGGT	110	*stx2*	Yuluo *et al.*, 2010
EVC-2	CTGCTGTCACAGTGACAAA			
Est-f	ATTTTTMTTTCTGTATTRTCTTCACCC	190	*est*	Hinenoya *et al*., 2009
Est-r	GGTACARGCAGGATT			
Elt-f	GGCGACAGATTATACCGTGC	450	*elt*	Hinenoya *et al.*, 2009
Elt-r	CGGTCTCTATATTCCCTGTT			

Antimicrobial susceptibility study

Susceptibility to different classes of antimicrobial agents was evaluated for *E. coli* using standard disc-diffusion method in Mueller Hinton agar using the inhibition-zone patterns. Antibiotic susceptibility and resistance patterns of *E. coli* against different antibiotics were measured according to the Kirby-Bauer method (Bauer *et al*., 1996). The zone diameter interpretative criteria of *E. coli* were used to classify isolates as susceptible, intermediate or resistant based on the standard interpretation table updated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2007). The discs containing the following amount of antibiotics were used : Penicillin-G 10 µg, Amoxycillin 30 µg , Rifampicin 5 µg, Gentamicin 10 µg, Cefuroxime 30 µg, tetracycline 30 µg, Erythromycin 15 µg, and Oxacillin 1 µg.

RESULTS AND DISCUSSION

Prevalence

The overall prevalence of *E. coli* causing diarrhea in calves of Bathan area was 57 %. However, several authors reported prevalence of *E. coli* associated diarrhea in calves which varied from 25.0 % to 49.8 % published from 2002 to 2014 (Malik *et al*., 2013; Ansari *et al*., 2014). But Nazir (2007) reported relatively higher (60%) prevalence. Samples positive to *E. coli* were grouped according to age, breed and sex (Table 2). A higher prevalence of *E. coli* causing diarrhea in calves (66.7%) were detected in calves of 6 days old, in calves of Sahiwal breed (85.7%) and in female calves 64.2%.

Table 2. Percentages of E. *coli* isolates in diarrheic calves according to different category of age, breed and sex.

Variables	Category level	No. of observation n = 100	No. of samples positive to *E. coli* (%)	95 % CI
Age	6 days	6	4 (66.7)	22.3-95.7
	Above 6 days to 1 month	61	39 (63.9)	50.6-75.8
	Above 1 month to 2 months	32	14 (43.8)	26.4-62.3
	2 months 15 days	1	0 (00)	0-97.5
Breed	Holstein Friesian cross	93	51 (54.8)	44.2-65.2
	Sahiwal cross	7	6 (85.7)	42.1-99.6
Sex	Male	47	23 (48.9)	34.1-63.9
	Female	53	34 (64.2)	49.8-76.9

CI=Confidence Interval

Detection of PCR products

Most EPEC strains have both bundle-forming pilus gene (*bfpA*) and *eaeA* gene, but in this study, the EPEC strains isolated were atypical in that they only contained the *eaeA* gene. PCR detection of 16 *E. coli* isolates showed that the detection rate of *eaeA* genes was 12.50% (Fig. 1, Table 3) which is correlated with Hur *et al*., 2013 in Korea who reported 13-17% (from diarrheic calves) of the same gene. From other studies the reported range of *eaeA* gene was 1.2% to 9.8% (Yuluo *et al*., 2010; Nguen *et al*., 2010; Salehi *et al*., 2011). Reasons for higher prevalence may be due to the sample size or number of experimented isolates, time of collections, age of the samples and age of the animals.

The differences of prevalence of virulence genes might be due to season, farm size, and number of animals on the farm, hygienic status, farm management practices, variation in sampling, variation in types of samples evaluated, and differences in detection methods.

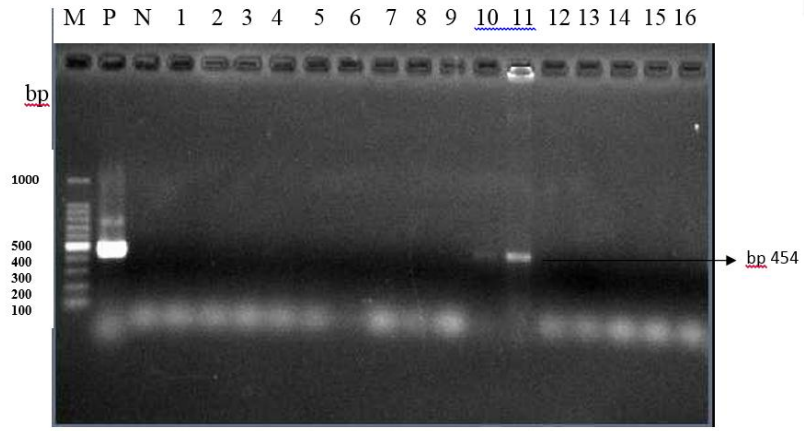

Fig. 1. Amplification of *eaeA* gene in *E. coli* isolates from diarrheic calves, M, Marker (1000 bp DNA ladder) P, positive control (*E. coli* O157 Sakai strain) and N, negative control (*E.coli* C600), Lane no 1-16 indicates sample no. (PCR products prepared from *E. coli* isolates).

Table 3. Detection rate of 6 pathogenic genes in *E. coli* isolates in diarrheic calves

Total no. of *E. coli* isolates used for PCR	Name of genes	Number of detected genes (n=16)	Percentage of 6 pathogenic genes (n=16)
16	*eaeA*	2.0	12.50
	bfpA	0.0	0.0
	stx1	0.0	0.0
	stx2	0.0	0.0
	est	0.0	0.0
	elt	0.0	0.0

Antimicrobial susceptibility testing

The antimicrobial susceptibility study of all the isolates against eight antibiotics used in this study revealed that most of the isolates were multidrug resistant and Oxacillin, Rifampicin and Penicillin has been shown to be 100% resistant followed by Erythromycin which is 80% resistant (Plate 1). Among all 8 antibiotics, Cefuroxime, although second generation of Cephalosporin, has shown to be moderately susceptible (30%) followed by Tetracycline (22%) and Gentamicin (12%) in this study. However, 2 *eaeA* gene *containing E. coli* showed 100% resistance to all antibiotics.

Hundred percent resistances to Penicillin was also reported by Malik *et al*., 2013 and the same in case of Erythromycin was reported by Nazir (2007) and Malik *et al.* (2013) in diarrheic calves. Malik *et al*. (2013) also reported Rifampicin as 100% resistant.

Rifampicin and Oxacillin are old drugs and is probably not used as veterinary drugs but it is still found resistant because of the fact that genomic plasticity of *E. coli* is very high. It can change its virulence properties very frequently. In addition, *E. coli* carry plasmid DNA and mobile genes and it can infect both human and animals. Due to its zoonotic potency these two drugs may have got resistance from human population. Acquisition of quick capability of transferring mobile genes from surrounding resistant strains of bacteria may occur through insertion, conjugation, transformation, transduction or other mechanisms, often facilitating the incorporation of the multiple resistance genes into the host's genome or plasmids.

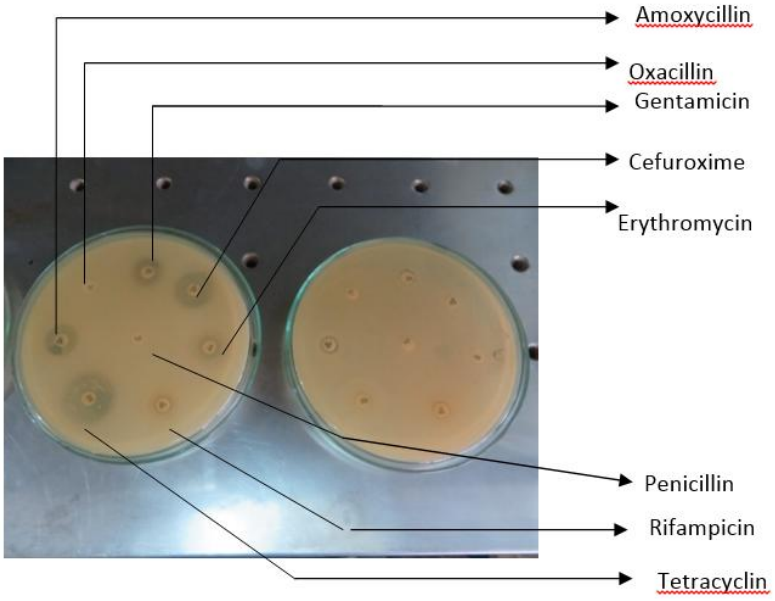

Plate 1. Antimicrobial susceptibility test of *E. coli* by agar disc diffusion method

In case of Amoxycillin and Tetracyclin 59.65% and 61.40% resistance were found respectively. Although 100% resistance in case of Tetracyclin was reported by Malik *et al*. (2013) and Ansari *et al*. (2014) reported more than 80%. On the contrary, 100% sensitivity to tetracycline was documented by Hossain *et al*. (2012). Higher resistance to amoxycillin was reported by Abd-Elrahman *et al*. (2011) and Ansari *et al*. (2014). Hossain *et al*. (2012) found Gentamicin as 100% resistant. While Malik *et al*. (2013) has found Gentamicin as moderately sensitive in this research Gentamicin was found moderately resistant Cefuroxime was found as moderately susceptible in this study which is supported by Orden *et al*. (1999) and Mahanti *et al*. (2014). But none of the drugs used in this study could be termed as single best in treating the *E. coli*causing diarrhea in calves.

This study stresses the importance of prevalence survey on the diarrheic *E. coli* isolated in diarrheic calves in Bathan region. So further investigation of the rest of the *E. coli* isolates is needed to detect any of 6 pathogenic genes to find out the real scenario of the prevalence of pathogenic genes existing in diarrheic calves.

REFERENCES

1. Abd-Elrahman AH (2011). Colibacillosis in newly born buffalo calves and role of lacteol fort in preventing recurrence of calf diarrhea. *Journal of Life Science* 8.
2. Abe CM, Trabulsi LR, Blanco J, Blanco M, Dahbi G, Blanco JE, Mora A, Franzolin MR, Taddei CR, Martinez MB , Piazza RMF, Elias WP (2009). Virulence features of atypical enteropathogenic *Escherichia coli* identified by the *eae*$^{+}$ EAF-negative *stx*$^{-}$ genetic profile. *Diagnostic Microbiology and Infectious Disease* 64**:** 357–365
3. Ansari RAIH, Rahman MM, Islam MJ, Das BC, Habib A, Belal SMSH and Islam K (2014). Prevalence and antimicrobial resistance profile of *Escherichia Coli* and *Salmonella* isolated from diarrheic calves. *Journal of Animal Health and Production* 2: 12–15.
4. Bauer AW, Perry DM and Kirby WM (1995). Single-disk antibiotic-sensitivity testing of *Staphylococci*: Ananalysis of technique and results. *Archives of Internal Medicine* 104: 208.
5. Bendali F, Bichet H, Schelcher F and Sanaa M (1999). Pattern of diarrhoea in newborn beef calves in southwest France. *Veterinary Research* 30: 61–74.
6. Blanco M, Blanco JE, Dahbi G, Mora A, Alonso MP, Varela G, Gadea MP , Schelotto F , Gonzalez EA , Blanco J (2006). Typing of intimin (eae) genes from enteropathogenic E.coli (EPEC) isolated from children with diarrhoea in Montevideo, Uruguay : identification of two novel intimin variants (lBandξR/b2B).*Journal of Medical Microbiology* 55: 1165–1174.
7. Bolton DJ (2011). Verocytotoxigenic (ShigaToxin-Producing) *E.coli* : virulence factors and pathogenicity in the farm to fork paradigm. *Foodborne Pathogens and Disease* 8: 357–365.
8. Carneiro LAM, Lins MC, Garcia FRA, Silva APS, Mauller PM, Alves B, Rosa ACP, Andrade JRC, Freitas-Almeida AC and Queiroz MLP (2006). Phenotypic and genotypic characterization of *E. coli* strains serogrouped as enteropathogenic *E.coli* (EPEC) isolated from pasteurised milk. *International Journal of Food Microbiology* 108: 15–21.
9. Cheesbrough M (1984). Medical Laboratory Manual for Tropical Countries. vol 11, Microbiology, pp.400-480.
10. CLSI (2007). Clinical and Laboratory Standards Institute (CLSI) guidelines. 27: Wane, PA, USA.
11. Cowan ST, Steel KJ, Barrow GI and Feltham RKA (1993). *Cowan and Steel's manual for the identification of medical bacteria*: Cambridge ; New York: Cambridge University Press.
12. Dulguer MV, Fabbricotti SH, Bando SY, Moreira-Filho CA, Fagundes-Neto U, Scaletsky IC (2003). A typical enteropathogenic *Escherichia coli* strains: phenotypic and genetic profiling reveals a strong association between enteroaggregative E. coli heat-stable enterotoxin and diarrhea. *The Journal of Infectious Disease* 188: 1685-1694.
13. Fecteau G, VanMetre DC, Pare J, Smith BP, Higgins R, Holmberg CA, Jang S and Guterbock W (1997). Bacteriological culture of blood from critically ill neonatal calves. *Canadian Veterinary Journal* 38: 95–100.
14. Hinenoya A, Naigita A, Ninomiya K, Asakura M, Shima K, Seto K, TsukamotoT, Ramamurthy T, Shah M. Faruque SM and Yamasaki S (2009). Prevalence and characteristics of cytolethal distendingtoxin-producing *Escherichia coli* from children with diarrhea in Japan. *Microbiology and Immunology* 53: 206–215.
15. Hornitzky M, Mercieca K, Bettelheim KA, Djordjevic SP (2005). Bovine feces from animals with gastrointestinal infections are a source of serologically diverse atypical enteropathogenic E.coli and Shigatoxin-producing E.coli strains that commonly possess intimin. *Applied and Environmental Microbiology* **71:** 3405–3412.
16. Hossain MK, Rahman M, Nahar A, Khair A and Alam MM (2012). Isolation and identification of diarrheagenic E*scherichia coli* causing colibacillosis in calf in selective areas of Bangladesh. *Bangladesh Journal of Veterinary Medicine* 11: 145-149.
17. Hur J, Jeon BW, Kim YJ, Oh IG and Lee JH (2013). *Escherichia coli* isolates from calf diarrhea in Korea and their virulent genetic characteristics. *The Journal of Veterinary Medical Science* 2: 519-22.
18. Kang SJ, Ryu SJ, Chae JS, Eo SK, Woo GJ and Lee JH (2004). Occurrence and characteristics of enterohemorrhagic *Escherichia coli* O157 in calves associated with diarrhoea. *Veterinary Microbiology* 98: 323–328.
19. Mahanti A, Samanta I, Bandyopadhyay S, Joardar SN, Dutta TK and Sar T K (2014). Isolation, molecular characterization and antibiotic resistance of Enterotoxigenic *E. coli* (ETEC) and Necrotoxigenic *E. coli* (NTEC) from healthy water buffalo. *Veterinarski arhiv* 84: 241-250.
20. Mailk S, Kumar A, Verma AK, Gupta MK, Sharma SD, Sharma AK and AnuRahal (2013). Incidence and Drug Resistance Pattern of Collibacillosis in Cattle and Buffalo Calves in Western Utter Pradesh in India. *Journal of Animal Health and Production* 1: 15–19.
21. Microbiology, Clinical and Laboratory Standards Institute (2011).Performance Standards for Antimicrobial Susceptibility Testing, Twenty first Informational Supplement M100-S21. Wayne, PA, USA.

22. Moller-Stray J, Eriksen HM, BruJheim T, Kapperud BA, Skeie A, Sunde M, Urdahl AM, Oygard B, Vold L (2012). Two outbreaks of diarrhea in nurseries in Norway after farm visits, April to May 2009. *Eurosurveilance* 17 : 947
23. Natato JP and Kaper JB (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews.* 11: 42-201.
24. Nazir KHM and Hussain N (2007). Plasmid profiles and antibiogram pattern of *Escherichia coli* isolates of calves feces and diarrhegenic stool of infants. *Journal of Bangladesh Social Agricultural Science and Technology* I: 149-152.
25. Nguyen TD, Vo TT and Vu-Khac H (2010). Virulence factors in *Escherichia coli* isolated from calves with diarrhea in Vietnam. *Journal of Veterinary Science* 12: 159-164.
26. Orden JA, Cid D, Ruiz-Santa-Quiteria JA, Garcia S, Martinez S and delaFeunte R (2002). Verotoxin-producing *E.coli* (VTEC) enteropathogenic *E.coli* (EPEC) and necro- toxigenic *E.coli* (NTEC) isolated from healthy cattle in Spain. *Journal of Appllied Microbiology* 93: 29–35.
27. Osek J, Gallien P and Protz D (2000). Characterization of Shiga toxin-producing Escherichia coli strains isolated from calves in Poland. Compendium on Immunology *Microbiology and Infectious Disease* 23: 267–276.
28. Paton JC and Paton AW (1998b). Pathogenesis and diagnosis of Shiga toxin-producing Escherichia coli infections. *Clinical Microbiology Reviews* 11: 450–479.
29. Salehi ZT, Badouei A, Mahdi, Brujeni N, Gholamreza, Madadgar and Omid (2011). Occurrence and characterization of enterohaemorrhagic isolates escherichia coli from diarrhoeic calves, department of microbiology, faculty of veterinary medicine, University of Tehran, Tehran, Iran.
30. Sivula NJ, Ames TR, Marsh WE and Werdin RE (1996). Descriptive epidemiology of morbidity and mortality in Minnesota dairy heifer calves. *Preventive Veterinary Medicine* 27: 155–171.
31. Svensson C, Lundborg K, EmanuelsonU and Olsson SO (2003). Morbidity in Swedish dairy calves from birth to 90 days of age and individualcalf-levelriskfactors for infectious diseases. *Preventive Veterinary Medicine* 58: 179–197.
32. Trabulsi LR, Keller R, Gomes TAT (2002). Typical and atypical enteropathogenic Escherichia coli. *Emerging Infectious Disease* 8: 508-513.
33. Trevejo RT, Barr MC and Robinson RA (2005). Important emerging bacterial zoonotic infections affecting the immunocompromised. *Veterinary Research* 36: 493–506.
34. Wieler LH, Schwanitz A, Vieler E, Busse B, Steinruck H, Kaper JB and Baljer G (1998). Virulence properties of Shiga toxin-producing *Escherichia coli* (STEC) strains of serogroup O118, a major group of STEC pathogens in calves. *Journal of Clinical Microbiology* 36: 1604–1607.
35. Yuluo WU, Hinenoya A, Taguchi T, Nagita A, Shima K, Tsukamoto T, Sugimoto N, Asakura M and Yamasaki S (2010). Distribution of Virulence Genes Related to Adhesins and Toxins in Shiga Toxin-Producing *Escherichia coli* Strains Isolated from Healthy Cattle and Diarrheal Patients in Japan. *The Journal of Veterinary Medical Science* 72: 589–597.