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RESEARCH ARTICLE

Isolation and Virulence Genes Characterization of Diarrheagenic *Escherichia coli* from Calves

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A B S T R A C T

Calf diarrhea due to *Escherichia coli (E. coli)* causes huge economic losses and possess an important veterinary health aspect. In current study, antibiotic profiling, molecular screening, and identification of *stx*-1, *eaeA*, *escV* and *bfpA* virulence genes of *E. coli* isolated from calves were carried out. Fecal samples from 28 diarrheagenic calves were taken, processed and cultured on MacConkey's agar, followed by identification using biochemical tests. Antibiotic susceptibility of the isolates was checked by disc diffusion method. For molecular characterization, polymerase chain reaction (PCR) was performed by using specific primers. Out of 28 samples collected from diarrheagenic calves, 83% samples were found positive for *E. coli* on the basis of biochemical profiling. Antimicrobial susceptibility against various antibiotics exhibited that most of the *E. coli* isolates were multi-drug resistant. All the isolates showed 100% resistance to rifampicin, erythromycin and oxacillin. The *E. coli* isolates were not resistant to gentamycin. Analysis through PCR showed that 10% of the isolates were positive for *stx*-1 gene, however, *bfpA*, *escV* and *eaeA* genes were not detected. Thus, *E. coli* is one of the key bacterium causing calve diarrhea and it was found that among all the virulent genes the *stx-1*plays a part in *E. coli* associated diarrheal infection in calves.

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INTRODUCTION

Calf diarrhea is a common disease in young animals causing major economic and health losses in livestock production worldwide. Data reported by National Animal Health Monitoring System from United States in 2007 revealed that almost 57% mortality among young calves is due to diarrhea (Cho and Yoon, 2014). Both infectious and non-infectious factors *i.e.* enteric pathogens and environmental factors are crucially involved in the development of calf diarrhea. The multi-factorial nature of this disease hinders the effective control of the disease in animal production (Foster and Smith, 2009). Diarrhea in calves is the most significant disease among developed and under developed countries. The definitive hosts including man, birds and animals are most susceptible for enteric diseases caused by *Eschericia coli (E. coli)* (Andrade *et al*., 2012: Islam *et al*., 2015).

Based on virulence mechanisms, Diarrheagenic *E. coli* (DEC) is classified as enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), entero-aggregative (EAggEC), diffusely adherent (DAEC), and enteropathogenic *E. coli* (EPEC) (Hashish *et al*., 2016). Among all strains, EPEC and EHEC affect the young calves between the ages of 2-8 weeks. EPEC is present in diarrheic and non- diarrheic calves producing characteristic lesions on intestinal epithelial cells brush borders and do not produce any kind of toxins (Abe *et al*., 2009). Intimin is a virulence protein expressed in *E. coli* cell surface that helps in adhesion of *E. coli* to its corresponding receptor, the translocated intimin receptor (tir) on the host cells (Stevens *et al*., 2006) and the gene encoding for it are termed as *eaeA* gene (Andrade *et al*., 2012).

Presence and absence of EAF plasmid (adherence factor) classify EPEC into two types as atypical EPEC (aEPEC) and typical EPEC (tEPEC). The typical EPEC has Adherence Factor Plasmid (EAF plasmid) that encodes *bfpA*, *Per* regulators and virulence genes (Scaletsky *et al*., 2010). Prevalence of aEPEC is more than tEPEC and is important in endemic disease and in

outbreaks (Ochoa and Contrera, 2011). The Shiga toxin producing *E. coli* (STEC) contains Shiga toxin 1 (*stx1*) or *stx2* and also known as Vero-toxin producing *E. coli* (VTEC). EHEC and EPEC are more important in humans as compare to animals (Schroeder *et al*., 2012).

Resistant *E. coli* strains induce persistent and severe infection when compared with antibiotic susceptible ones (Lakshmidevi *et al*., 2014). It is also described that most prevalent resistances were observed against Ampicillin, Tetracycline, Streptomycin and Sulfonamides (Kolenda *et al*., 2015). Extensive use of antibiotics in developing countries is thought to be the main cause of resistance. This problem may also affect the isolates from adults with diarrhea. The current research was planned in order to detect presence of DEC and molecular characterization of virulence genes along with antibiotic sensitivity pattern study.

MATERIALS AND METHODS

Purification and Bio characterization: The present study was performed at Institute of Microbiology, University of Agriculture Faisalabad. Twenty-eight samples from the rectum of diarrheagenic calves under three months of age were collected and diluted with 150mM of normal saline followed by centrifugation at 4000 RPM for 5 minutes. Supernatant was inoculated on MacConkey's agar medium plates and incubation was done at 37˚C for 24 hrs. Isolates were identified for cultural and morphological features and preserved in lactose broth comprising 20% glycerol. Further confirmation of *E. coli* was done using the biochemical tests *i.e.* Indole, Methyl red, Voges-Proskauer and Triple Sugar Iron test (Brisse *et al*., 2006).

Antibiotic sensitivity profiling: Susceptibility of *E. coli* to commonly used antibiotics was done through standard disc diffusion method and zone of inhibitions were measured. Isolates were designated as susceptible, intermediate and resistant according to the Clinical and Laboratory Standards Institute (CLSI) standards (Bokhari *et al*., 2013). Antimicrobial agents tested were Penicillin (10µg), Cefuroxime (30µg), Tetracycline (30µg), Oxacillin (1µg), Gentamicin (10µg), Erythromycin (15µg), Amoxicillin (30µg) and Rifampicin (5µg) (Silva and Mendonca, 2012). *E. coli* (ATCC # 25922) strain was included as a quality control.

Molecular characterization of *E. coli* from diarrheagenic calves: DNA from *E. coli* was extracted with Phenol/Chloroform Method (Bergallo *et al*., 2006). *E. coli* was enriched in Brain Heart Infusion Broth for overnight incubation at 37ºC followed by centrifugation at 8000 rpm. Pellet was used for DNA extraction. DNA concentration was measured using Thermo Scientific NanoDrop™ Spectrophotometer. All the positive isolates were characterized by using PCR for *stx*-1, *eaeA*, *bfpA* and *escV* genes using specific set of primers (Table 1). Amplified PCR products were electrophoresed on agarose gel (John *et al*., 2008).

RESULTS

Prevalence of *E. coli* in diarrheagenic calves: Based on colony morphology on MacConkey's agar medium followed by biochemical characterization, twenty-five (25) samples (83%) were found positive for the presence of *E. coli* collected from the diarrheagenic calves. Fig. 1 represents the percentage distribution of all samples collected from diarrheagenic calves.

Purification and microscopic examination of *E. coli* isolates from calves: After 24 hrs incubation on MacConkey's agar medium, *E. coli* colonies appeared pink due to lactose fermentation on MacConkey's agar medium as shown in Fig. 2. Under microscope, *E. coli* appeared as pink colored Gram-negative rods.

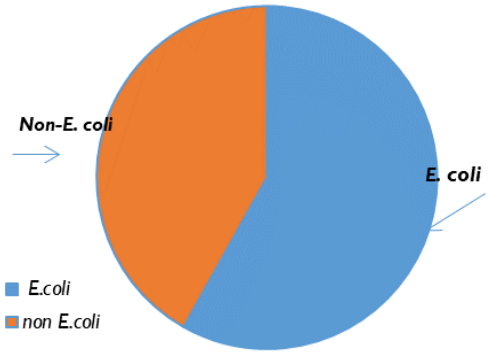

Fig. 1: Percentage positivity of *E. coli* isolated from fecal samples of calves.

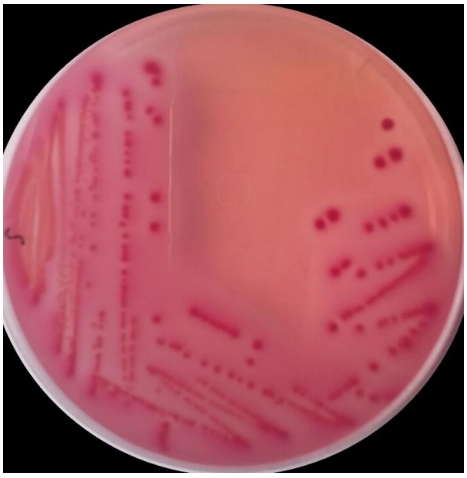

Fig. 2: Primary culture of *E. coli* on MacConkey's agar isolated from fecal samples of calves. The *E. coli* colonies appear in deep red color on MacConkey's agar.

Table 1: Primer sequences used for amplification of *eaeA*, *bfpA*, *Escv* and *Stx1* genes of *E. coli*

Primer	5' ⟶ 3'	Amplicon size (bp)	Gene	Reference
eaeA1	AAACAGGTGAAACTGTTGCC CTCTGCAGATTAACCTCTGC	453	*eaeA*	Yuluo *et al.*, 2010
eaeA2				
bfpA	FAATGGTGCTTGCTTGCGGCTTGCTGC	323	*bfpA*	Yuluo *et al.*, 2010
bfpA	R GCCGCTTTTATCCAACCTGGTA			
Escv	FATTCTGGCTCTCTTCTTCTTTATGGCTG	543	*Escv*	Muller, Greune *et al.*, 2007
Escv	RCGTCCCCTTTTACAAACTTCATCGC			
KS 7	CCCGGATCCATGAAAAAAACATTATTAATAGC	282	*Stx1*	
KS 8	CCCGAATTCAGCTATTCTGAGTCAACG			

Bio characterization of *E. coli* isolated from calves: A total of 25 out of 28 *E. coli* isolates were positive for catalase, Indole and Methyl red tests. All these 25 isolates also showed fermentation of dextrose, lactose and/or sucrosein TSI medium.

Antimicrobial susceptibility of *E. coli* isolated from diarrheagenic calves: Antibiotic susceptibility pattern of *E. coli* to different antibiotics observed on Muller Hinton agar is shown in Fig. 3 (A & B). The percentage resistance of isolates against seven different antibiotics is shown in Table 2. The *E. coli* anti-biogram revealed 100% resistance against Oxacillin, Rifampicin and Erythromycin followed by Amoxicillin (60%). It showed less resistance to Cefotaxime (30%) and Tigecycline (33%). No resistance was observed against Gentamycin (0%). Fig. 4 represents the histogram showing results for antibiotic susceptibility trend of seven different antibiotics against *E. coli* isolates.

PCR amplification of *E. coli* isolated from calves: A total of 25 samples were selected for the amplification of *eaeA*, *stx*-1, *bfpA* and *escV* genes using the specific set of primers for each gene mentioned in Table 1. DNA concentration of all the 25 isolates positive for *E. coli* was analyzed by Nano drop spectrophotometer. The entire PCR product for each gene was subjected to gel electrophoresis. Among the 25 samples that were subjected to PCR for amplification, only two samples (10%) showed a band of 282 base pairs indicating the presence of *stx*-1 gene (Fig. 5). No bands were observed for the remaining three genes (*eaeA*, *bfpA* and *escV*) in any of the 25 samples.

DISCUSSION

Based on the virulence factors, DEC is classified into different groups named as EHEC, ETEC, DAEC, EIEC, EPEC and enteroaggregative *E. coli* (EAggEC).

Primary identification through culture characteristics and biochemical tests confirmed 25 (83%) positive isolates for *E. coli*. This high prevalence rate found was higher when compared to studies by Islam *et al*. (2015). A comparatively lesser percentage 25 to 49.8% was also reported by Mailk *et al*. (2013) while relatively higher percentage (60%) was reported by Ansari *et al*. (2014). This difference in prevalence might be due to the selection of animals for sampling, health and immune status of calves, study area and the farm management practices.

Antibiotics are not advised for treatment of mild and acute diarrhea. Majority of the isolates showed drug resistance to seven antibiotics used in this study. A complete (100%) resistance was observed against Rifampicin and Oxacillin followed by Erythromycin (90%) and Ampicillin (60%). It was reported that *E. coli* is resistant to Ampicillin (41%) (Kelly, 2015). Average susceptibility for Tigecycline was also noticed and only 33% resistance was shown to it, while other report showed comparatively higher resistance to it (Badouei *et al*., 2014). No resistance was observed for Gentamycin. Resistance to Erythromycin (90%) was compatible with previous studies (85%). In diarrheic calves, complete resistance to Erythromycin (97.6%), was reported (Pourtaghi & Sodagari, 2016). Resistance to Rifampicin was also studied (Nazir and Hussain, 2007).

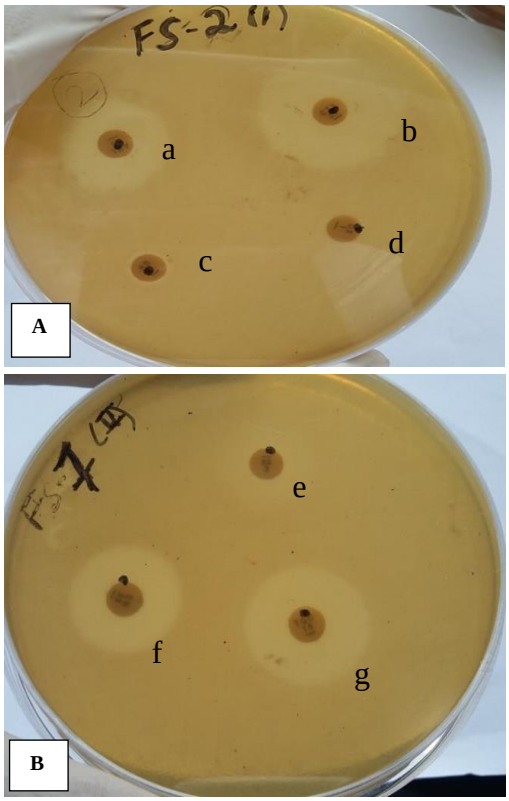

Fig. 3: (A & B) Antimicrobial sensitivity pattern of *E. coli isolated from calves* against selected antimicrobial classes. The zones of inhibition indicate the isolates were either resistant or sensitive to the specific drug according to CLSI criteria. (a) Cefotaxime (b) Amoxicillin (c) Oxacilline (d) Rifampicin (e) Erythromycin (f) Gentamycin and (g) Tigecycline.

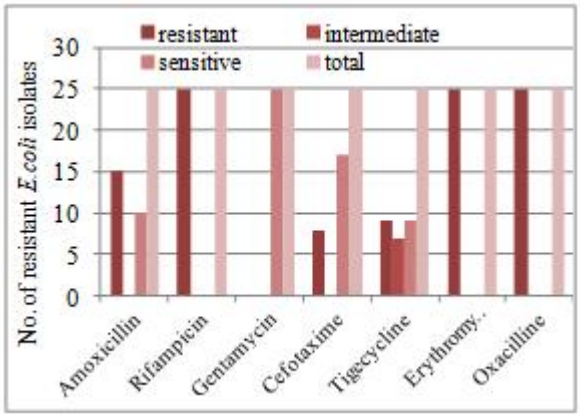

Fig. 4: Graphical representation of antibiotic sensitivity against *E. coli* isolates from calves.

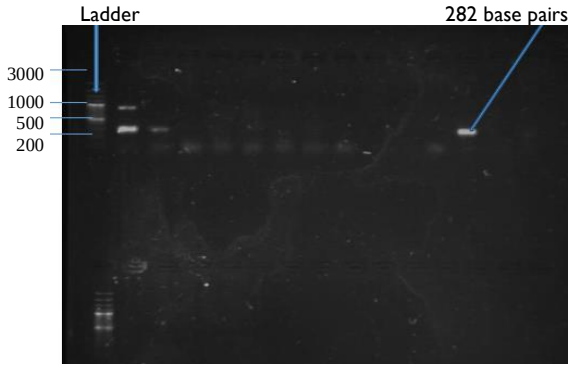

Fig. 5: Agarose gel electrophoresis of PCR products obtained for *stx*-1 gene. On left hand side gene ladder is indicated while on the right hand side *stx*- gene of 282 base pair size is shown.

Table 2: Percentage resistance of *E. coli* isolates from calves to selected antibiotics

Sr. No.	Antibiotic	Disc conc.	No of *E. coli* isolates resistant	Resistance (%)
1	Amoxicillin	30µg	15	60
2	Rifampicin	5µg	25	100
3	Gentamycin	10µg	0	0
4	Cefotaxime	30µg	8	30
5	Tigecycline	15µg	9	33
6	Erythromycin	15µg	25	100
7	Oxacilline	1µg	25	100

A 60% resistance was found in case of Amoxicillin, supported by a report in which almost same results were reported, i.e., 59.65% resistance (Kelly, 2015). *E. coli* was also found resistant to Rifampicin and Oxacillin, even though both are not used in veterinary practice. This resistance might be due to high genomic plasticity of *E. coli* as it can vary its virulence properties quite often. Moreover, it also carries mobile genes and plasmid DNA as it can infect both humans and animals. Transduction, insertion, conjugation, transformation are possible mechanisms for acquiring of mobile genes from the surrounding resistant strains thus facilitating resistance genes to get incorporate into the plasmids or host's genome (Younis *et al*., 2009).

Most EPEC strains have both bundle-forming pilus gene (*bfpA*) and *eaeA* gene, but the current study deals with aEPEC which did not harbor *bfpA* gene. The *bfpA* gene was not found in the present study which correlates with the results of Islam *et al*. (2015). The investigation of aEPEC in other studies confirmed that the *bfpA* gene is present only in tEPEC (Nguyen *et al.,* 2010; Yuluo *et al.,* 2010; Salehi *et al.,* 2011). In the present study, gene *eaeA* could not be amplified, whereas, the prevalence of *eaeA* gene was reported as 1.2 to 9.8%, when compared with other studies (Nguyen *et al.,* 2010, Yuluo *et al.,* 2010; Salehi *et al.,* 2011). This difference in the results might be due to time of collection, number of the samples or age of the animals. In the present study, the targeted *stx*-1 gene was amplified in 2 of the selected samples (10%), whereas it was reported to be 29 28, 18 and 4.3% in another study (Yulou *et al.,* 2010). Other possible reasons for differences in virulence genes might be due to variation in sampling, number of animals, farm size, management practices, and season on the farm, hygienic status, farm, and variation in types of samples and differences in detection methods (Hossain *et al*., 2014).

Conclusions: Neonatal calve diarrhea caused by *E. coli* is an important disease of calves in early life. It has been found that *stx-1* gene contribute to the virulence of *E. coli* infection in calves. Also, this study focuses on the demand for more epidemiological survey and detection of other pathogenic genes among diarrheic *E. coli* to find out the current scenario for pathogenic genes prevalence in diarrheic calves.

Authors contribution: MIA designed the experiment and write the paper. AP executed the experiments. FRA, AAB, SA, and SUR contributed in proof reading, formatting and writing.

REFERENCES

Abe CM, Trabulsi LR, Blanco J, *et al*., 2009. Virulence features of atypical Enteropathogenic *Escherichia coli* identified by the eae+ EAF-negative stx− genetic profile. Diagn Microbiol Infect 64:357-65.

Andrade GI, Coura FM, Santos ELS, *et al*., 2012. Identification of virulence factors by multiplex PCR in *Escherichia coli* isolated from calves in Minas Gerais, Brazil. Trop Anim Health Prod 44:1783-90.

Ansari RAIH, Rahman MM, Islam MJ, *et al*., 2014. Prevalence and antimicrobial resistance profile of *Escherichia coli* and *Salmonella* isolated from diarrheic calves. J Anim Heal Prod 2:12-5.

Badouei MA, Lotfollahzadeh S, Arman M, *et al*., 2014. Prevalence and resistance profiles of enteropathogenic and shiga toxin producing *Escherichia coli* in diarrheic calve in Mashhad and Garmsar districts, Iran. Avicenna J Clin Microb Infec 1:22-80.

Bergallo M, Costa C, Gribaudo G, *et al*., 2006. Evaluation of six methods for extraction and purification of viral DNA from urine and serum samples. New Microbiol 29:111-9.

Bokhari H, Shah MA, Asad S, *et al*., 2013. *Escherichia coli* pathotypes in Pakistan from consecutive floods in 2010 and 2011. Am J Trop Med Hyg 88:519-25.

Brisse S, Grimont F and Grimont PD, 2006. The genus Klebsiella taxonomic history and structure. Prokaryotes 6:159-96.

Cho YI and Yoon KJ, 2014. An overview of calf diarrhea-infectious etiology, diagnosis, and intervention. J Vet Sci 15:1-17.

Foster DM and Smith GW, 2009. Pathophysiology of diarrhea in calves. Vet Clin North Am Food Anim Pract 25:13-36.

Hashish EA, Damaty HM, Tartor YH, *et al.*, 2016. Epidemiological study of diarrheagenic *Escherichia coli* virulence genes in newborn calves. Pak Vet J 36: 54-8.

Hossain MK, Rahman M, Nahar A, *et al*., 2014. Isolation and identification of diarrheagenic *Escherichia coli* causing colibacillosis in calf in selective areas of Bangladesh. Bangal J Vet Med 11:145-9.

Islam AKMA, Rahman M, Nahar A, *et al*., 2015. Investigation of pathogenic *Escherichia coli* from diarrheic calves in selective Area of Bangladesh. Bangal J Vet Med 13:45-51.

John HL, Hur J and Stien BD, 2008. Occurrence and characteristics of Enterohemorrhagic *Escherichia coli* O26 and O111 in calves associated with diarrhea. Vet J 176:205-9.

Kolenda R, Burdukiewicz M and Schierack P, 2015. A systematic review and meta-analysis of the epidemiology of pathogenic *Escherichia coli*of calves and the role of calves as reservoirs for human pathogenic *E. coli*. Front Cell Infect Microbiol 5:23-30.

Lakshmidevi N, Al-hetar KY and Kavishankar GB, 2014. Extended spectrum beta lactamase producing extra-intestinal pathogenic escherichia coli (ESBL-ExPEC). J Pharm Biomed Sci 4:343-55.

Mailk S, Kumar A, Verma AK, *et al*., 2013. Incidence and drug resistance pattern of collibacillosis in cattle and buffalo calves in Western Utter Pradesh in India. J Anim Heal Prod 1:15-9.

Nazir KHM and Hussain N, 2007. Plasmid profiles and antibiogram pattern of *Escherichia coli* isolates of calves' feces and diarrhegenic stool of infants. J Bangl Soc Agric Sci Technol 4:149-52.

Nguyen TV, Van PL, Huy CL, *et al*., 2010. Detection and characterization of diarrhoeagenic *Escherichia coli* from young children in Hanoi, Vietnam. J Clin Microbiol 43:755-60.

Ochoa TJ and Contrera CA, 2011. Enteropathogenic *E. coli* (EPEC) infection in children. Curr Opin Infect Dis 24:478-83.

Kelly P, 2015. Infectious Diarrhoea. Medicine 43:253-8.

Pourtaghi H and Sodagari HR, 2016. Antimicrobial resistance of entrotoxigenic and non-entrotoxigenic *Escherichia coli* isolated from diarrheic calves in Iran. Int J Enteric Pathog 4:1-5.

Scaletsky ICA, Souza TB, Aranda KRS, *et al*., 2010. Genetic elements associated with antimicrobial resistance in Enteropathogenic *Escherichia coli* (EPEC) from Brazil. BMC Microbiol 10:25-31.

Salehi TZ, Badouei MA and Gohari IM, 2011. Molecular detection and antibacterial susceptibility of enteropathogenic *Escherichia coli* (EPEC) and shiga toxigenic *Escherichia coli* (STEC) strains isolated from healthy and diarrhoeic dogs. Comp Clin Pathol 20:585-9.

Schroeder ME, Bounpheng MA, Rodgers S, *et al.,* 2012. Development and performance evaluation of calf diarrhea pathogen nucleic acid purification and detection workflow. J Vet Diag Invest 24:945-53.

Silva GJD and Mendonca N, 2012. Association between antimicrobial resistance and virulence in *Escherichia coli*. Virulence 3:18-28.

Stevens JM, Galyov EE and Stevens MP, 2006. Actin-dependent movement of bacterial pathogens. Nat Rev Microbiol 4:91-5.

Younis EE, Ahmed AM, El-Khodery SA, *et al*., 2009. Molecular screening and risk factors of enterotoxigenic *Escherichia coli* and *Salmonella* spp. in diarrheic neonatal calves in Egypt. Res Vet Sci 87:373-9.

Yulou WU, Hinenoya A, Taguchi T, *et al*., 2010. Distribution of virulence genes related to adhesions and toxins in Shiga toxin-producing *Escherichia coli* strains isolated from healthy cattle and diarrheal patients in Japan. J Vet Med Sci 72:589-97.