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ISOLATION AND IDENTIFICATION OF DIARRHEAGENIC *Escherichia coli* CAUSING COLIBACILLOSIS IN CALF IN SELECTIVE AREAS OF BANGLADESH

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ABSTRACT

This study was carried out to isolate, identify and characterize *Escherichia coli* from diarrheic calves, their prevalence and antibiotic sensitivity pattern during the period from November 2011 to December 2012. For this purpose 100 diarrheic fecal samples were collected from four different areas of Bangladesh. The samples were aseptically collected and brought to the laboratory of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh. The organisms were identified as *E. coli* on the basis of cultural, morphological and biochemical characteristics. Further the isolated bacteria were confirmed by amplification of *E. coli* specific 16sRNA gene. The antibiotic resistant pattern of the isolates was also performed. Out of 100 samples 49 (49%) were found to be positive for *E. coli*. The highest prevalence was found in Shahjadpur, Sirajganj (53.33%) while lowest was in BAU, Mymensingh (40%). The antibiogram study revealed that all isolates were resistant against ampicillin, erythromycin, azithromycin and streptomycin where erythromycin was 100% resistant, whereas 100% sensitivity was found to tetracycline and gentamicin which might be the best choice of drug for the treatment of diarrheagenic *E. coli* causing colibacillosis in calves.

Key words: Colibacillosis, *Escherichia coli,* isolation, identification, prevalence, antibiotic sensitivity.

INTRODUCTION

In the national economy of Bangladesh, the livestock sector plays an important role with the contribution of 2.51% in 2012-2013 to the agricultural GDP. Besides, it contributes more than 6.20 percent of the total foreign exchange earning through exporting hides and skins, leather products, hooves bone and others. The estimated cattle population is 23.20 million in Bangladesh in 2011-2012. It provides meat and milk for human consumption as high quality protein, hides and draft power for ploughing, rural roads and power transport, threshing, oil seed crushing, dung as fuel and manure.

Escherichia coli (E. coli) is a gram-negative, aerobic, rod-shaped, flagellated, motile, oxidase negative, non-spore forming, small sized (2-3x 0.6μ) and toxin producer (endotoxin) and are classified under the family Enterobacteriaceae (Buxton and Fraser, 1977). The colon bacillus popularly known as *E. coli* was first isolated from the normal faecal material of a 2-3 days old newborn baby and subsequently from young calves in 1885 by Escherich (Buxton and Fraser, 1977).

E. coli is widely distributed in nature, being present in soil, surface water, animal and human faeces. *E. coli* produces septicemia and diarrhea in a wide range of hosts including man, avian and animals. Calves are the most vulnerable to *E. coli* infection. Two age groups appear to be of calves of 1-3 days of age and of 3-8 weeks of old are more susceptible. Symptoms include diarrhea, a rise in body temperature, general weakness, dehydrated and lack of appetite. This is soon followed by coma and death within a few hours. In older animals are tendency of infection to localize itself in the joints of survivors. Lesions include enlarged, hemorrhagic spleens and the accumulation of synovial fluid and sometimes pus in affected joints (Radostits *et al.* 2000). Pathogenicity of *E. coli* strains are due to the presence of one or more virulence factors including invasiveness factors like invasins, heat labile, heat stable enterotoxins, verotoxins and colonization factors or adhesins (Kaper *et al.,* 2004). Pathogenic *E. coli* are divided into two types- Enteropathogenic *E. coli* and Uropathogenic *E. coli.* Further pathogenic *E. coli* are grouped into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli (EIEC),* enteroaggregative *E. coli* (EAggEC), enterohemorrhagic *E. coli* (EHEC). Calf diarrhoea is being controlled primarily following the maintenance of strict hygienic and sanitary measures. The economic aspect of diarrheal diseases in calves and their mortality and morbidity is a matter of great concern to the livestock owners. *E. coli* can be isolated, identified and characterized using cultural, morphological, biochemical, serological and molecular techniques.

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Cultural characterization of *E. coli* by using different media and biochemical characterization by observing variable reaction to different sugars and chemicals are considered as the basic tools for their identification (Hasina, 2006).

Antibiotics are widely used for the treatment of diarrheic. In the context of Bangladesh for many years antibiotic is randomly used for the treatment purpose. Subsequent use of more powerful and/or new antibiotics is being needed to defeat the genetically altered bacterial population. As a result, generations of bacterial strains are being developed which are multidrug resistant and new types of antibiotics are required for the prevention and control of diseases (Nazir, 2004). *E. coli* has become resistant to many antimicrobials through the acquisition of mobile drug resistance genes. The incidence of multiple antibiotic resistant *E. coli* strains has been increasing. Considering the above situations, the present research work was designed with the following objectives: i) To isolate, identify and characterize *E. coli* causing colibacillosis in calf, ii) To study the prevalence of colibacillosis in calf and the antimicrobial sensitivity pattern of the isolated bacteria.

MATERIAL AND METHODS:

The research work was conducted in the Department of Medicine and the Department of Microbiology & Hygiene, Bangladesh Agricultural University (BAU), Mymensingh-2202 during the period of November 2011 to December 2012.

Collection of samples

A total of 100 faecal samples were collected aseptically from the rectum of diarrhoeic calves from Tangail, Delduar, Sirajganja and Mymensingh district. After collection, the samples were transported to the laboratory of Microbiology and Hygiene, faculty of Veterinary Science, BAU as soon as possible for bacteriological examinations and further characterization.

Isolation of *E coli*

Standard methods were followed for the isolation and identification of *E. coli* (Cowan, 1985). Nutrient broth (NB) was used for primary culture of *E. coli* organisms from the faecal samples followed by culture into differential and selective media such as Eosin Methylene blue (EMB) and MacConky (MC) agar to identify the bacteria.

Identification and characterization of *E. coli*

The isolated bacteria was identified as *E. coli* by observing their cultural characteristics, morphology by Gram's stain, biochemical tests, motility test and finally by amplification of E. coli specific 16s RNA gene. After primary isolation of the *E. coli*, the bacteria were inoculated onto Mac Conkey and EMB agar which were subjected to biochemical tests- five basic sugar fermentation tests and motility tests according to the method described by Cheesbrough, (1984) and Cowan (1985) respectively

Detection of 16s rRNA gene of *E. coli* by PCR

Extraction of DNA from the Escherichia coli was carried out by conventional boiling and rapid cooling method. In brief, a single colony of *E coli* was resuspended in 100 µl of nuclease free water and boiled for 10 minutes and immediately cooled on icebox followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was collected which was used as template DNA.

Details of the oligonucleotide primers used for the amplification of 16S rRNA are illustrated in Table (1). PCR reaction mixture consisted of 12.5 µl of 2 × PCR master mixtures (Promega), 10 pmol primer of each and 2 µl of genomic DNA in a final volume of 25 µl adjusted by nuclease free water. The cycling conditions consisted of initial denaturation at 95°C for 5 min., followed by 30 cycles of 94° C for 1 min., 55°C for 45 seconds min. and 72°C for 1 min., with final extension at 72°C for 7 min. The amplified products were electrophoresed into 1.8% agarose gel at 100 volt visualized under Gel doc/UV trans-illuminator.

Antibiogram Study

Ten different commercially available antimicrobial discs were used for antibiogram study as described in Table-1. Susceptibility and resistance of different antibiotics were measured in vitro by employing the Kirby-Bauer method (Bauer *et al.,* 1959). The plates were then examined the diameters of the zone of inhibition. Isolates were classified as susceptible, intermediate and resistant categories based on the standard interpretation table (Table 2) updated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2011).

Table 1. Primer sequences, target genes, locations within gene and predicted sizes of amplified products of *E. coli* specific 16S rRNA

Primer code	Oligonucleotide sequence (5`-3`)	Target gene	Location within gene	Amplicon size (bp)
16s-F 16s-R	GAC CTC GGT TTA GTT CAC AGA CAC ACG CTG ACG CTG ACC A	16S rRNA	4267278-4267845	485

Table 2. Antibiotic disc with their concentration

Name of Antimicrobial agent	Disc concentration (µg/disc)
Ampicillin	25
Chloramphenicol	30
Tetracycline	30
Erythromycin	15
Azithromycin	15
Streptomycin	10
Gentamicin	10
Nalidixic acid	30
Ciprofloxacin	5 µg
Norfloxacin	10 µg

µg = Microgram,

RESULT AND DISCUSSION

This research was undertaken to isolate and identify *E. coli* from the diarrhoeic calves. Standard methods were followed for the isolation and identification of *E. coli* (Cowan, 1985).

Bacteria of the Enterobacteriaceae are of important pathogens causing intestinal and systemic illness of humans and other animals. Recent outbreaks of gastrointestinal diseases focused public attention on one of the more widely known members, *Escherichia coli* and the potential problems with strains of this organism as Colibacillosis in calf. Traditional approaches for characterization of *E. coli* have relied on cultural techniques and many selective differential media have been developed. Usually lactose fermentation is used for differentiation, sodium lauryl sulfate or bile salts are used as a selective agent. The emergence of DNA technology has opened new possibilities for development of methods with improved selectivity for *E. coli* specific genes coding different proteins (Gannon et al. 1997) and toxins.

Out of 100 fecal samples from the diarrheic calves 49 were positive for bacterial isolation on to media like MacConkey agar and EMB. All the 49 isolates produced positive reaction to lactose fermentation on MacConkey agar plate, metallic green sheen colonies on EMB plates. These findings were in correlation with the presentation of Cowan (1993) and John Barnes *et al.,* (2003). The prevalence of *E. coli* infection was previously reported as 13.71% in diarrhoeic calves in Bangladesh (Debnath *et al.,* 1990). The present study found relatively higher prevalence of *E. coli* infection different areas of Bangladesh causing colibacillosis in calves in compare to the previous. The higher prevalence of *E. coli* infection and differences of prevalence in different areas might be due to lack of hygiene, overcrowding, lack of post milking teat dipping, lack of effective preventive measures, differences in sample size and also diagnostic techniques used for the detection of *E. coli*.

Gram's staining revealed that all the isolates are Gram negative, non spore forming bacilli. These findings were in correlation with the presentation of John Barnes *et al.* (2003).

The organisms are motile observed under microscope. All the isolated organisms fermented five basic sugars lactose, dextrose, mannitol, sucrose and maltose producing acid with gas.

Acid production was indicated by the colour change from red to orange yellow color with gas production manifested by the appearance of gas bubbles in the inverted Durham's tubes.

The PCR was subsequently used to screen 100 faecal samples collected from diarrhoeic calf. This was done for confirmation of their identity as *E. coli* in calf colibacillosis. The *E. coli* specific 16s RNA gene was amplified from all isolates. Thus the PCR results showed that it can be used for monitoring the colibacillosis in calf and other livestock. At the genomic level, optimized PCR assay was able to successfully amplify the expected size 585 bp fragment from the genomic DNA of *E. coli.* The 16s RNA, PCR is a sensitive, specific, and rapid method for the confirmation of the *E. coli* (Sabat *et al*., 2000). By amplification of a 16S rRNA gene that effectively distinguishes *E. coli* from the closely related bacteria.

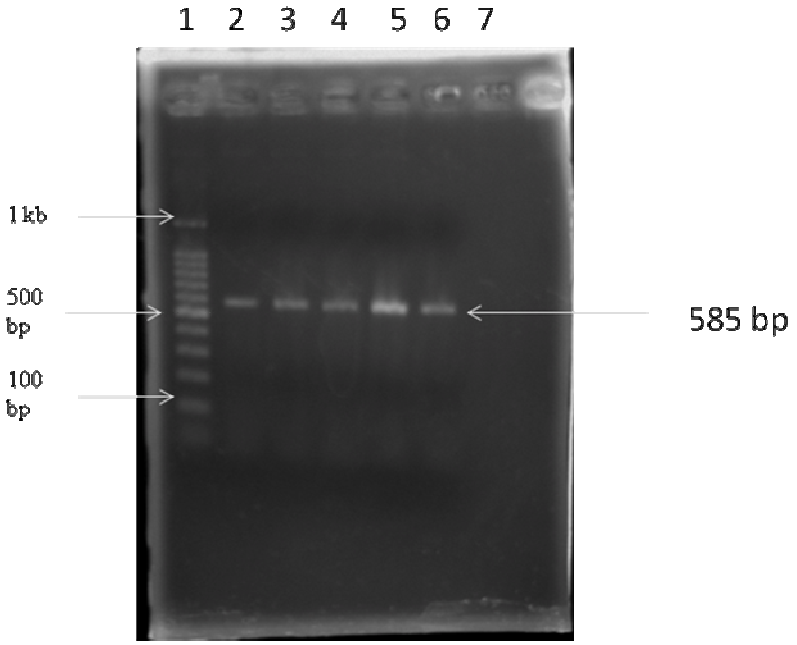

Figure 1. Amplification of 16s RNA from isolated *E.coli.* Lane-1; 100 bp ladder and lane 2-6; positive samples, lane-7, negative control.

Table 3. Prevalence of *Escherichia coli* in diarrhoeic calves

SI. No.	Sources and Location	Sample size	Number of calves positive for *E. coli*	Prevalence (%)
1	Delduar, Tangail	30	14	46.67
2	Tangail Sadar, Tangail	25	13	52.00
3	Shahjadpur, Sirajganj	30	16	53.33
4	BAU Vet. Clinics, Mymensingh	15	6	40.00
	Total	100	49	49.00

The difference of prevalence in different locations was not statistically significant ($p \leq 0.05$).

Antibiogram study of the isolates indicated that out of 49 isolates, most of the isolates were highly susceptible to tetracycline, chloranphenical, norfloxacine and gentamicin, moderately sensitive to nalidixic acid, whereas 100% sensitive to tetracycline and gentamycin(Table 3). All the isolates were highly resistant to ampicillin, azythromycin and streptomycin except erythromycin to which 100% resistance were observed. The results indicate that most of the antibiotics except erythromycin showed more or less intermediate to their action in the treatment of colibacillosis.

Table 4. Results of Antimicrobial susceptibility test using various isolates of *Escherichia coli*

Sensitivity pattern	Number (%) of isolated *E. coli* sensitive to various antibiotics									
	AMP	C	TE	E	AZM	S	GN	NA	CIP	NOR
Susceptible	4	38	38	0	0	0	36	30	16	36
	(8.16)	(77.55)	(77.55)	(0.00)	(0.00)	(0.00)	(73.46)	(61.22)	(32.65)	(73.46)
Intermediately	8	8	7	0	3	13	13	16	28	5
resistant	(16.32)	(16.32)	(14.28)	(0.00)	(6.12)	(26.53)	(26.53)	(32.65)	(57.15)	(10.21)
Resistant	37	3	5	49	46	36	0	3	5	8
	(75.51)	(6.12)	(10.21)	(100)	(93.85)	(73.46)	(0.00)	(6.12)	(10.21)	(16.32)

AMP-Ampicillin, C-Chloramphenicol, TE-Tetracycline, E-Erthromycin, AZM-Azithromycin, S-Streptomycin, GN-Gentamycin, NA- Nalidixic acid, CIP-Ciprofloxacin and NOR-Norfloxacin

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