Original Research

Isolation, Antibiogram and Molecular Characterization of Enterotoxigenic *Escherichia coli* from Diarrheic Bovine Calves

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Abstract

A total of 117 fecal samples were collected from diarrheic calves from various regions of Gujarat. Out of total fecal samples screened, 89.74 % samples were found to be positive for E. coli by PCR assay using species specific phoA gene. Region wise prevalence of E. coli were found to be highest in Surat (100%). Age wise, E. coli infection was highly prevalent in age group-I (1-10 days) i.e. 41.88%. In vitro antibiotic resistant pattern showed high resistance against pefloxacin (68.57%), oxytetracycline (65.71%) and erythromycin (64.76%) while colistin resistance was observed in 10.47% isolates. All the isolates were screened for the presence of virulence associated genes by PCR assay. Out of 105 isolates, 29.52%, 26.66% and 7.61% isolates harboured STa, F5 and LT-1gene, respectively. Whereas, 11.42%, 3.80% 2.85%, 1.90% isolates possess genes in combination of F5 and STa, F5 and LT1, STa and LT1 and F5, STa and LT-1, respectively.

Key words: Calf Scour, ETEC, Neonatal Calf Diarrhea, PCR

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Introduction

Livestock is an essential part of the agricultural system in India and plays an important role in national economy as well as in socio-economic development of millions of rural livelihoods of India. Neonatal calf diarrhea is one of the most common diseases in young animals, causing huge economic and productive losses to bovine and dairy industry worldwide (Cho and Yoon, 2014). Disease is a multifactorial complex syndrome including infectious (Bacteria, Virus, Protozoa) as well as non-infectious factors related to the animal *viz.* immunological and nutritional status, the environment or the management as mentioned by Izzo *et al*. (2011). Neonatal calves are at the greatest risk of diarrhea in a first month of their life. Infectious diarrhea is the most significant cause of morbidity and mortality in neonatal dairy calves throughout the

world and it can be caused by many pathogens including bacteria like *E. coli* and *Salmonella* as reported by Acha *et al.* (2004); viruses like *Rotavirus* and *Coronavirus* and some extent by *Bovine viral diarrhea virus, Parvovirus, Astrovirus, Enterovirus* by Izzo *et al*. (2011); and protozoa like *Cryptosporidium parvum* and *Eimeria* spp. as reported by Izzo *et al*. (2011).

Escherichia coli (*E. coli*) is a gram negative, rod-shaped, motile, facultative anaerobic, non-spore forming member of *Enterobacteriaceae* family found in the gastrointestinal tract of warm-blooded animals and humans (Markey *et al.*, 2013). Eosin methylene blue medium (EMB) is a selective medium for *E. coli* isolation. *E. coli* can be classified into six patho groups viz. Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Entero invasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Enterohaemorrhagic *E. coli* (EHEC) and Shiga toxin producing *E. coli* (STEC) as mentioned by Quinn *et al.* (2011).

Among all above pathogroups, ETEC strain is mainly responsible for neonatal calf diarrhea in young animals via production of variety virulence factors. *E. coli* is transmitted by ingestion of contaminated feed, water, soil, faeces and direct contact from one person to another as mentioned by Ashraf *et al.* (2018). ETEC most commonly produce theF5adhesin antigen and the heat-stable (STa or STb) and/or heat-labile (LT-1 or LT-2) enterotoxins as described by Markey *et al.* (2013). STa and STb are only type of toxins usually produced by bovine and porcine ETEC, respectively. Pathogenic *E. coli* strains are responsible for pathogenicity by allowing them to colonize the host's small intestinal mucosa through its unique colonization factors as well as producing STa and LT-1 enterotoxins. Thus, avoiding the immune response and stimulating the deleterious inflammatory response to produce diarrhea reported by Younis *et al.* (2009) and Croxen and Finlay (2010). The acute form of disease is characterized by progressive dehydration and death, sometimes in as few as 12 hrs. Considering to the role of ETEC in development of neonatal calf diarrhea, the present study was aimed to isolate *E. coli* from diarrheic calves culturally, confirming it is by PCR, develop the antibiogram and characterization of isolates for various virulence genes.

Materials and Methods

A total of 117 fecal samples were collected from calves of cattle and buffalo of 0-8 weeks of age. Samples were collected from farms of various region viz. Anand (n=31), Surat (n= 68) and Junagadh(n=18). The fecal samples were collected as per the method of Yilmaz (2016). The information such as age, sex and location of the calves were recorded. Diarrheal fecal samples of calves were inoculated on MacConkey agar (MA) and EMB for primarily identification of *E. coli*. Hemolytic activity of *E. coli* isolates was observed on 5% sheep blood agar as per Markey *et al*. (2013). The pure culture of *E. coli* isolates was stored in brain heart infusion (BHI) slants for further identification and other biochemical tests. The DNA was extracted by snap chill method. Briefly, the suspension of organisms was made in 100µl of Milli-Q water by picking

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up a typical colony in a 200µl PCR tube. The suspension was heated at 95°C for 15 minutes followed by sudden chill and all cell debris were removed by centrifugation at 10,000 rpm for 1 minute, and 3µl of the supernatant was used as a template DNA. The *E. coli* isolates also confirmed by PCR by using *phoA*primer (Fig. 1). The isolates were subjected to *in vitro* antibiotic sensitivity testing as per the method of Bauer *et al.* (1966). Isolates were tested against commonly used antibiotics were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

All the *E. coli* isolates were characterized for viz. *F5, STa* and *LT-1* genes using different sets of primers as given in Table 1. Steps and thermocycling condition of primer for above genes as per Table 2. Quantity and concentration of various components used for PCR were as per Table 3. The amplified PCR products were analysed by agarose gel electrophoresis on 2% agarose gel and visualized under UV light by a gel documentation system (Syngene, India).

Table 1: List of oligonucleotide primers used in PCR

Target Genes	Name of Primers	Sequences (5'-3')	Expected product size (bp)	Reference
phoA	phoA F	CGATTCTGGAAATGGCAAAAG	720	Abdulgayeid *et al.* (2015)
	phoA R	CGTGATCAGCGGTGACTATGAC		
F5	F5 F	TATTATCTTAGGTGGTATGG	314	El-Seedy *et al.* (2016)
	F5 R	GGTATCCTTTAGCAGCAGTATTTC		
STa	ST F	GCTAATGTTGGCAATTTTTATTTCTGTA	190	Pourtaghi *et al.* (2015)
	ST R	AGGATTACAACAAAGTTCACAGCAGTAA		
LT-1	LT F	AGCAGGTTTCCCACCGGATCACCA	132	Wani *et al.* (2013)
	LT R	GTGCTCAGATTCTGGGTCTC		

Table 2: Steps and conditions of thermocycling for detection and characterization of *E. coli* isolates

Sr. No.	Steps		*phoA* gene	*F5* gene	*STa* gene	*LT-1* gene
1	Initial denaturation	Temperature	94ºC	94ºC	94ºC	94ºC
		Time	5min	5min	5min	5min
2	Denaturation	Temperature	94ºC	94ºC	94ºC	94ºC
		Time	45sec	60sec	60sec	30sec
3	Annealing	Temperature	56ºC	55ºC	55ºC	56ºC
		Time	45sec	60sec	60sec	60sec
4	Extension	Temperature	72ºC	72ºC	72ºC	72ºC
		Time	60sec	90sec	90sec	90sec
5	Final Extension	Temperature	72ºC	72ºC	72ºC	72ºC
		Time	8min	10min	10min	8min
Cycles			30	35	35	30

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Table 3: Quantity and concentration of various components of PCR reaction mixture

S. No.	Components	Volume	Concentration
1	2 X PCR Master Mix	12.50µl	2X
2	Forward Primer (10pmol/µl)	1.00µl	10pmole
3	Reverse Primer (10pmol/µl)	1.00µl	10pmole
4	Template DNA	3.00µl	-
5	Nuclease Free Water	7.50µl	-
Total		**25.00µl**	-

Results and Discussion

Overall Prevalence of *E. coli*

In the present study, out of total 117 samples screened, 89.74% (105/117) were found to be positive for *E. coli* by cultural characteristics and confirmed by PCR assay. Similar prevalence rate was reported El-Seedy *et al.* (2016) and Tarekegn and Molla (2017) whereas in contrast lower prevalence were reported by Gebregiorgis and Tessema (2016), Dawod *et al.* (2016) and Hakim *et al.* (2017). The difference in prevalence of *E. coli* in the above studies might be attributed to the variations in environmental and managemental conditions such as insufficient and/or poor-quality colostrum feeding/intake, gaps in management specifically calf handling practices including inadequate nutrition, exposure to severe environment, insufficient attention to the newborn calf, or a combination of these are often involved in scours outbreaks.

Region Wise Prevalence of *E. coli*

A total 105 isolates which includes 29 (93.54%) isolates from Anand, 68 (100%) from Surat and 8 (44.44%) from Junagadh. Difference in prevalence in various regions might be due to the difference in climatic conditions, sample size, management practices, personal hygiene, the age at which sample was collected and the farm size.

Age and Sex Wise Prevalence of *E. coli*

The isolation rates of *E. coli* decreased with increasing age of calves. The highest percentage of *E. coli* positive samples was detected in age group-I (1-10 days) i.e. 41.88% followed by 24.76% in age group-II (11-20 days), 20.95% in age group-III (21-30 days) and 8.57% in age group-IV (31-60 days), respectively. Villarroel (2009) reported that neonatal calves under 1 week of age are particularly more susceptible because the normal flora of the intestine is not fully established, have a naive immune system and also receptors for the adhesions of *E. coli* are present during the first week of life of the calves. Dawod *et al.*, (2016); Gebregiorgis and Tessema (2016) reported that calves of age group-I (1-10 days) are most affected

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clinically might be due to an inadequate, poor quality of colostrum and delay in first colostrum feeding, which leads to failure of transfer of passive immunity is an important reason.

Cultural Isolation and Identification of *E. coli* Isolates

Based on cultural, morphological and staining characters, 105 isolates were identified as *E. coli*. Based on cultural characters like lactose fermenting colonies on MLA and preliminary identification by primary tests viz. 3% KOH, catalase and oxidase test, the isolates were identified as *E. coli*. Further, the isolates were confirmed as *E. coli* on EMB agar.

Overall Antibacterial Resistant Pattern of *E. coli*

The total 105 isolates obtained from diarrheal calves were subjected for *In vitro* sensitivity against 22 different antibiotics. The result of individual isolate to various drugs was interpreted as per manufacturer's instructions (HiMedia Pvt. Ltd., Mumbai) and the results are presented in Table 4.

Table 4: Overall results of antibiotic susceptibility testing of *E. coli* isolates

Name of Antibiotic	No. of isolates		
(Code)	Sensitive (%)	Intermediate (%)	Resistant (%)
Amikacin (AK, 30 mcg)	57 (54.29)	23 (21.90)	25 (23.81)
Amoxyclav (AMC, 30 mcg)	53 (50.48)	21 (20.00)	31 (29.52)
Ampicillin (AMP, 25 mcg)	26 (24.76)	15 (14.29)	64 (60.95)
Cefixime (CFM, 5 mcg)	51 (48.57)	24 (22.86)	30 (28.57)
Cefoparazone (CPZ, 75 mcg)	48 (45.71)	21 (20.00)	36 (34.29)
Cefotaxime (CTX, 30 mcg)	32 (30.48)	6 (5.71)	67 (63.81)
Ceftriaxone (CTR, 30 mcg)	60 (57.14)	16 (15.24)	29 (27.62)
Cephalothin (CEP, 30 mcg)	24 (22.86)	25 (23.81)	56 (53.33)
Chloramphenicol (C, 30 mcg)	100 (95.24)	0 (0.00)	5 (4.76)
Ciprofloxacin (CIP, 5 mcg)	58 (55.24)	25 (23.81)	22 (20.95)
Colistin (CL, 10 mcg)	94 (89.52)	0 (0.00)	11 (10.47)
Co-trimoxazole (COT, 25 mcg)	65 (61.90)	2 (1.90)	38 (36.19)
Enrofloxacin (EX, 10 mcg)	56 (53.33)	22 (20.95)	27 (25.71)
Erythromycin (E, 15 mcg)	0 (0)	37 (35.24)	68 (64.76)
Gentamicin (GEN, 10 mcg)	84 (80.00)	11 (10.48)	10 (9.52)
Moxifloxacin (MO, 5 mcg)	39 (37.14)	0 (0.00)	66 (62.86)
Oxytetracycline (OTC, 30 mcg)	15 (14.29)	21 (20.00)	69 (65.71)
Pefloxacin (PF, 5 mcg)	33 (31.43)	0 (0.00)	72 (68.57)
Spectinomycin (SPT, 100 mcg)	70 (66.67)	30 (28.57)	5 (4.76)
Streptomycin (S, 10 mcg)	31 (29.52)	30 (28.57)	44 (41.90)
Sulphadiazine (SZ, 300 mcg)	54 (51.43)	0 (0.00)	51 (48.57)
Tetracycline (TE, 30 mcg)	38 (36.19)	9 (8.57)	58 (55.24)

The findings in present study are in agreement with earlier studies by Wani *et al.* (2013), Hossain *et al.* (2014) and Dawod *et al.* (2016) reported high resistant against ampicillin 78%, 75.51% and 74%,

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respectively. In contrast to the present study, Abdulgayeid *et al.* (2015) and Abubaker *et al.* (2015) reported high resistant against oxytetracycline (91.57%) and erythromycin (84.70%), respectively.

Prevalence of drug resistant in *E. coli* against amikacin, amoxiclav, ampicillin, cefotaxime, colistin, erythromycin, gentamicin, moxifloxacin, oxytetracycline and sulphadiazine was variable in all the three regions as per Table 5.

Table 5: Region wise Antibacterial Resistant Pattern among *E. coli* Isolates

Name of Antibiotics (Code)	Isolates of Region		
	Anand (%) (29)	Surat (%) (68)	Junagadh (%) (8)
Amikacin (AK, 30 mcg)	3.45	32.35	25
Amoxyclav (AMC, 30 mcg)	31.03	32.35	0
Ampicillin (AMP, 25 mcg)	48.28	67.65	50
Cefixime (CFM, 5 mcg)	27.59	29.41	25
Cefoparazone (CPZ, 75 mcg)	34.48	33.82	37.5
Cefotaxime (CTX, 30 mcg)	58.62	64.71	75
Ceftriaxone (CTR, 30 mcg)	31.03	26.47	25
Cephalothin (CEP, 30 mcg)	48.28	54.41	62.5
Chloramphenicol (C, 30 mcg)	3.45	5.88	0
Ciprofloxacin (CIP, 5 mcg)	20.69	20.59	25
Colistin (CL, 10 mcg)	6.9	11.76	12.5
Co-trimoxazole (COT, 25 mcg)	41.38	33.82	37.5
Enrofloxacin (EX, 10 mcg)	31.03	23.53	25
Erythromycin (E, 15 mcg)	55.17	66.18	87.5
Gentamicin (GEN, 10 mcg)	13.79	5.88	25
Moxifloxacin (MO, 5 mcg)	65.52	58.82	87.5
Oxytetracycline (OTC, 30 mcg)	79.31	63.24	37.5
Pefloxacin (PF, 5 mcg)	75.86	64.71	75
Spectinomycin (SPT, 100 mcg)	3.45	5.88	0
Streptomycin (S, 10 mcg)	31.03	47.06	37.5
Sulphadiazine (SZ, 30 mcg)	37.93	54.41	37.5
Tetracycline (TE, 30 mcg)	62.07	52.94	50

Whereas prevalence of drug resistant in *E. coli* to amikacin (3.45%), chloramphenicol (3.45%) and colistin (6.90%) of Anand region are very less as compared with other two regions might be due to the minimum used of these antibiotics and awareness of owner regarding antibiotic resistant. The *E. coli* isolates of Surat were highly resistant to ampicillin (67.65%), erythromycin (66.18%), cefotaxime and pefloxacin (64.71% each), oxytetracycline (63.24%), moxifloxacin (58.82%), cephalothin (54.41%) and tetracycline (52.94%).

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Arya (2004) studied antibiogram pattern of *E. coli* isolated from diarrheic calves of the same region. She reported high resistance against amikacin (91.20%), enrofloxacin (84.61%), ampicillin (72.52%) and tetracycline (54.04%). In contrary to results of Arya (2004), the present study revealed that *E. coli* isolates were less resistant to amikacin (23.81%) and enrofloxacin (25.71%). This variation in resistant pattern might be due to less frequent use of these particular antibiotics during the past 15 years. Strikingly, the resistant against colistin was also recorded in isolates obtained from Junagadh (12.50%), Surat (11.76%) and Anand (6.90%) region. The colistin is used as a last resort of antibiotic for the treatment of Gram-negative bacterial infection and resistance towards colistin in present study speculate a future where there will not be any antibacterial in nearer future for the treatment of such infections in animals and humans.

In the present study, the highest rate of resistant has been recorded against antibiotic most commonly used either as feed additives or as curative agents in farm animals or for treatment in human medicine. This warrants restriction on the use of antibiotics as feed additives and rational use of antibiotic for infections in man and animals.

Molecular Characterization of *E. coli* Isolates Obtained from Diarrheic Calves for Virulence Factor

The PCR was performed for detection of *F5*, *STa* and *LT-1*gene of ETECusing specific primer pair, which yielded expected product size of 314bp, 190bp and 132bp, respectively as shown in Fig. 2, 3 and 4. The result of present study is in the agreement of Ok *et al.* (2009) as they detected 18.90% of *F5* and *Sta* each in *E. coli* isolates. Wani *et al.* (2013) recorded *Sta*and *LT-1* at the rate of 17.39% and 73.91%, respectively. In contrary to present study, Arya (2004), Shams *et al.* (2012), Wani *et al.* (2013) detected lower prevalence of *F5, Sta* and *LT-1* genes among the *E. coli* isolates. There were numbers of isolate possessed more than one virulence associated genes. Wani *et al.* (2013) and Abubaker *et al.* (2015) detected 2 and 5 isolates which carried genes for both *Sta* and *LT-1*, respectively.

In the present study, it was found that 12 (11.42%), 4 (3.80%), 3(2.85%) and 2 (1.90%) isolates possess *F5* and *STa, F5* and *LT-1, Sta* and *LT-1*and *F5, Sta* and *LT*-1 genes, respectively as per Table 6. Abubaker *et al.* (2015) detected 5 isolates possessing both *Sta* and *LT-1*. In contrast to present study, Shahrani *et al.* (2014) detected 100% presence of *F5* and *LT* virulence associated genes and detected 8 (4.46%) isolates having all three genes *F5, Sta* and *LT* enterotoxin genes.

Table 6: Overall prevalence of virulence associated gene/s among *E. coli* isolates

Total No. of Isolates	No. of Isolates Possess Virulence Associated Genes						
	F5	***STa***	***LT-1***	***F5+Sta***	***F5+LT-1***	***STa+ LT-1***	***F5+STa+LT-1***
105	28 (26.66%)	31 (29.52%)	08 (7.61%)	12 (11.42%)	4 (3.80%)	3 (2.85%)	2 (1.9%)

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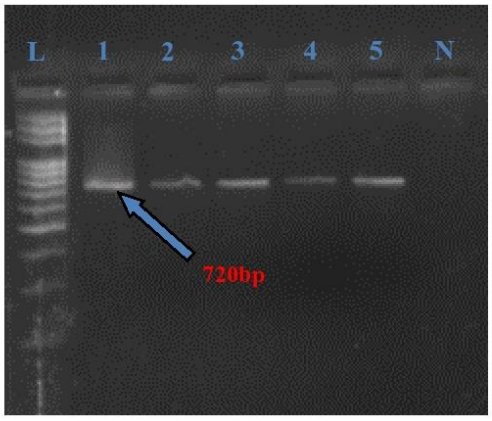

Figure-1: Partial length amplification of *phoA* gene of *E. coli*. Lanes: M: 3kb DNA ladder (Fermentas). 1-4: *E. coli* isolates. P: Positive control. N: Negative control Numbers on the side depict size of PCR amplicon in base pairs.

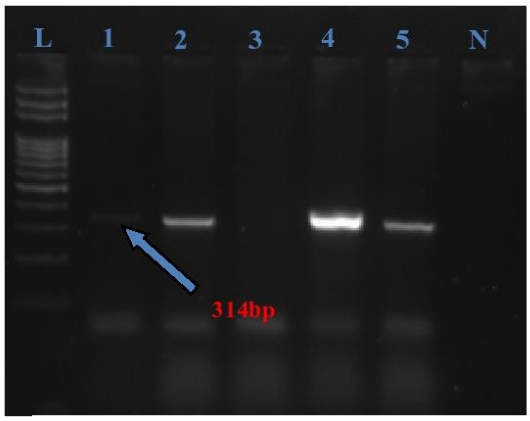

Figure- 2: Partial length amplification of *F5* gene of *E. coli*. Lanes: M: 3kb DNA ladder (Fermentas). 1-4: *E. coli* isolates. P: Positive control. N: Negative control Numbers on the side depict size of PCR amplicon in base pairs.

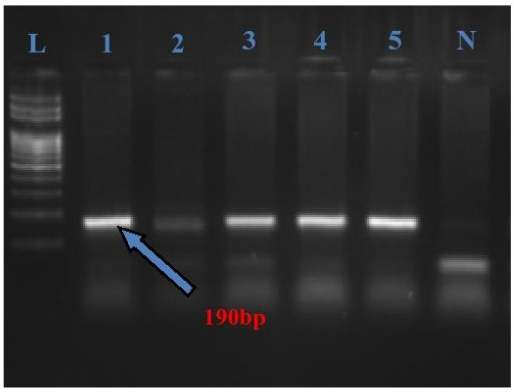

Figure- 3: Partial length amplification of *STa* gene of *E. coli*. Lanes: M: 3kb DNA ladder (Fermentas). 1-4: *E. coli* isolates. P: Positive control. N: Negative control Numbers on the side depict size of PCR amplicon in base pairs.

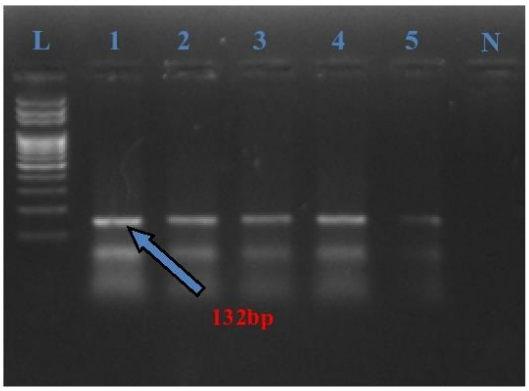

Figure- 4: Partial length amplification of *LT-1* gene of *E. coli*. Lanes: M: 3kb DNA ladder (Fermentas). 1-4: *E. coli* isolates. P: Positive control. N: Negative control Numbers on the side depict size of PCR amplicon in base pairs.

Conclusion

From the present study, it can be concluded that overall prevalence of *E. coli* is 89.74%. Prevalence of *E. coli* is higher in Surat (100%) and Anand (93.54%). The age wise, highest prevalence was recorded in 1-10 days age. The isolates were highly resistance to pefloxacin (68.57%), oxytetracycline (65.71%), erythromycin (64.76%), cefotaxime (63.81%) moxifloxacin (62.86%), ampicillin (60.95%), tetracycline (55.24%) and cephalothin (53.33%). Among all isolates, colistin resistance was observed in 10.47% isolates, which warrants the indiscriminate use of colistin. The highest prevalent virulence associated genes were *Sta* (29.52%) and *F5* (26.66%).

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Conflict of Interest

The authors declare no conflict of interest.

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