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Research Article

ISOLATION AND MOLECULAR CHARACTERIZATION OF *E. COLI* FROM BOVINE WITH EMPHASIS ON VIRULENCE DETERMINANTS AND ANTIMICROBIAL RESISTANCE

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Abstract: The present study was undertaken to isolate, detect the virulence factors, antimicrobial susceptibility and antimicrobial resistance genes in of *Escherichia coli* from cases of bovine mastitic milk samples and faecal samples from diarrhoeic cows and buffaloes. Total 173 samples comprising milk samples from cases of bovine mastitis (n=128) and faecal samples (n=45) from diarrhoeic cows and buffaloes were subjected for isolation of *E. coli*. Out of these samples, 53 *E. coli* isolates were recovered with overall prevalence of 30.64 per cent which includes 29.69 per cent (38/128) from milk samples and 33.33 per cent (15/45) from faecal samples. All the 53 isolates revealed characteristic features of *E. coli*, which were Gram negative bacilli, produced pink lactose fermenting colonies on Mac Conkey agar and characteristic greenish metallic sheen on eosin methylene blue agar. Biochemical characterization of all the isolates revealed characteristic IMViC pattern *viz*., indole and M.R positive and V.P and citrate negative. For Genotypic confirmation, all the 53 presumptive isolates of *E. coli* were subjected to PCR using species-specific 16S rRNA gene (ECO-1) amplification. All the 53 isolates successfully amplified 585 bp amplicon from the genomic DNA which confirmed them as a *E. coli*. Out of all the 53 *E. coli* isolates screened, 13 (24.52%) isolates were found positive for ehly gene, in which amplicon size of 432 bp was detected and none of the isolates was found to possess genes for intimin protein. All the 53 *E. coli* isolates were subjected to antibiotic susceptibility test using 18 different antibiotics. The result revealed different susceptibility patterns. Out of 53 isolates of *E. coli*, 42 (79.72%) isolates showed sensitivity to Chloramphenicol, 35 (66.36%) to Colistin, 31 (58.15%) to Cefepime, 30 (56.36%) to Amoxyclav, 29 (54.72%) to each Ceftazidime-clavulanic acid, Gentamicin, Carbapenem and Ampicillin/sulbactam, 28 (52.83%) to each Enrofloxacin and Aztreonam, 24 (45.28%) to Cefotaxime-clavulanic acid, 23 (43.40%) to Ceftriaxone-Sulbactam, 19 (35.85%) to Doxycycline, 17 (32.08%) to Ceftriaxone, 15 (28.30%) to Cefotaxime, 14 (27.79%) to Streptomycin, 11 (20.75%) to Ceftazidime and 7 (13.79%) to Vancomycin.

Keywords: *Escherichia coli, Antimicrobial resistance, Virulence genes, Polymerase chain reaction*

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Introduction

Mastitis is one of the most hazardous and costly infectious diseases of the dairy industry, affecting animal welfare and having potential public health implications if untreated or inadequately treated milk is consumed. Mastitis is the inflammation of the mammary gland caused by traumatic injury, chemical irritation, but most often, infections is caused by bacteria. Cows with mastitis may exhibit clinical signs associated with inflammation, bacterial toxemia and occasionally septic shock. Mastitis results in increased costs for the producer from veterinary expenses, production loss and increased labor [1]. Farm environment is an important source of coliform mastitis and *E. coli* is most isolated coliform organism [2, 3].

Diarrhoea is also one of the major community health hazards both for man and animal which is caused by bacteria, virus, fungus, protozoa, helminths, chemical agents, clay, sands, nutritional deficiency factors, indigestion, managemental factors, hepatic cirrhosis and other toxic factors. These factors act singly or in combination to produce diarrhoea complex [4]. Among them, *E. coli* is a major cause of diarrhoea in farm animals. *E. coli* is generally a commensal but includes some highly pathogenic strains carrying additional genes in plasmids or the chromosome. Based on the genes the pathogenic strains are divided into pathotypes *viz*. Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffusely Adherent *E. coli* (DAEC). Each of these pathotypes has different virulence attributes that help them to cause infections by different mechanisms which result in variable clinical symptoms [5].

E. coli is a Gram negative, rod-shaped, facultative anaerobic bacterium. Pathogenic *E. coli* can be categorized based on serogroups, pathogenic mechanisms, variation in epidemiology and different interaction with the intestinal mucosa, clinical symptoms or virulence factors [6].

However, most of the time, antimicrobial therapy does not adhere to previous pathogen susceptibility tests, and as a result, antimicrobial-resistant bacteria have emerged as a result of improper use or inadequate doses of antimicrobials [7]. *E. coli* isolated from bovine mastitis were resistant to at least one of the antimicrobial classes [8]. Moreover, multidrug resistant *E. coli* have been reported from bovine mastitis [9]. It has been reported that antimicrobial resistant bacteria cause more severe and persistent form of mastitis compared to those caused by antibiotic susceptible counterparts. Furthermore, occurrence of multidrug resistant virulent *E. coli* in bovine mastitis is a critical public health concern which threatens the public of transmitting zoonoses and food toxin infections [10].

Considering the losses resulting from mastitis and diarrhoea, the recognition of *E. coli* as highly adaptive bacteria in different ecological niches, treatment failure and probability of prevalence of ESBL producing *E. coli*, the present study was planned.

Materials and Methods

Isolation and Phenotypic Identification of *E. coli*

A total 173 samples comprising milk samples from cases of bovine mastitis (n=128) and faecal samples (n=45) from diarrhoeic cows and buffaloes belonging to various places of Banaskantha, Mahesana, Sabarkantha and Patan districts of Gujarat were collected aseptically in sterilized vials. All the faecal samples and milk samples were streaked on the Brain heart infusion agar (BHI) plates for primary isolation of *E. coli*.

Table-1 *Details of primers for amplification of ECO-1 gene employed in PCR*

Gene designated	Primer sequence (5'- 3')		Size of amplified products (bp)	Ref
ECO-1	Forward	GACCTCGGTTTAGTTCACAGA	585	[12]
	Reverse	CACACGCTGACGCTGACCA		

Table-2 *Steps and conditions of thermal cycling for ECO-1gene based PCR*

Primers	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
ECO-1	94°C	94°C	53°C	72°C	72°C
	5 min.	30 sec.	1 min.	1 min.	8 min.
		Repeated for 30 cycles			

Table-3 *Details of primers for amplification eaeA and ehly genes of E. coli*

Gene designated	Primer sequence (5'- 3')		Size of amplified products (bp)	Ref
eaeA	Forward	TGCGGCACAACAGGCGGCGA	629	[13]
	Reverse	CGGTCGCCGCACCAGGATTC		
Ehly	Forward	CAATGCAGATGCAGATACCG	432	[14]
	Reverse	CAGAGATGTCGTTGCAGCAG		

Table-4 *Steps and conditions of thermal cycling for eaeA and ehly genes in PCR*

Primers	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
eaeA gene	94°C	94°C	50°C	72°C	72°C
	5 min.	45 sec.	45 sec.	45 sec.	4 min.
		Repeated for 30 cycles			
ehly gene	94°C	94°C	64°C*	72°C	
	4 min.	40 sec.	45 sec.	1 min.	
		Repeated for 10 cycles			72°C
		94°C	54°C	72°C	10 min.
		45 sec.	45 sec.	1 min.	
		Repeated for 30 cycles			

*Touchdown polymerase chain reaction (PCR) was used for the amplification of *ehly* gene. Annealing at 64°C for 45s with an incremental decrease in 1°C per cycle.

The plates were incubated in an inverted position at 37°C for 24 hrs. After incubation, the culture smear of the isolate was prepared on microscopic glass slide and stained with Gram's method of staining. Gram negative with short rods and coccobacilli morphologically similar to *E. coli* were transferred and streaked on MacConkey's agar for differentiation of lactose fermenting and non-lactose fermenting bacteria and incubated for 18-24 h at 37°C. Only lactose fermenting bacteria having pinkish colour colonies were sub cultured on Eosin Methylene Blue (EMB)agar and incubated aerobically at 37°C overnight. Colonies showing metallic sheen were identified and considered as presumptive *E. coli*. Such colonies were confirmed using arrays of biochemical tests (IMViC).

Extraction of Bacterial DNA

Genomic DNA was extracted from freshly grown culture by boiling method as per the method described by [11]. In brief, 3 to 5 bacterial colonies were picked up and suspended in 50 µl deionized water followed by boiling for 5 min and centrifuging at 10000 rpm for 1 min. The supernatant was then transferred and used as the DNA template for further molecular characterization.

Genotypic confirmation of *E. coli*

All the probable *E. coli* isolates were subjected to molecular confirmation using species-specific 16S rRNA gene (*ECO-1*) amplification.

PCR was carried out in final reaction volume of 20 µl in a thin walled 200 µl PCR tubes using a Nexus Mastercycler (Eppendorf) under the following conditions.

Electrophoresis and Gel Documentation

To confirm the targeted PCR amplification, Mixture of PCR product 5 µl from each tube was mixed with 2.0µl of 6X gel loading dye from each tube was loaded in separate wells on the submerged gel. Then it was electrophoresed on 1.5 per cent agarose gel along with 100bp DNA Ladder (Gelpilot) at constant 80 V for 30-45 minutes in 1X TAE buffer. The amplified product was visualized as a single compact band of expected size under Gel Documentation System (Invitrogen life technology E-Gel imaging system, Israel). The molecular sizes of the DNA bands were analyzed in relation to molecular weight DNA ladder.

Virulence genes characterisation of *E. coli*

The analysis of virulence factors of *E. coli* isolates comprised the detection of Intimin (*eaeA*) and Haemolysin (*ehly*) genes by PCR.

Antibiotic sensitivity patterns of *E. coli* isolates

The antibiotic sensitivity patterns of *E. coli* isolates were determined using a disk diffusion test [15]. For susceptibility test, 4-5 pure colonies were transfered to 5 ml Brain Heart Infusion (BHI) broth and incubated at 37°C until optical density reached 0.1 at 620 nm. A sterile cotton swab dipped in standardized inoculum was used to streak the entire agar surface of the Mueller and Hinton (MH) agar plate swabbing three times and the plate was kept at 60° angle after each streaking. The inoculum was allowed to dry for 10 minutes with lid in place. Monodiscs (Hi Media, Pvt. Ltd., Mumbai) of antibiotics *viz*., Cefepime (30 mcg), Ceftazidime (30 mcg), Ceftazidime-clavulanic acid (30/10 mcg), Cefotaxime (30 mcg), Cefotaxime-clavulanic acid (30/10 mcg), Ceftriaxone (30 mcg), Ceftriaxone/salbactam (30/15 mcg), Aztreonam (30 mcg), Gentamicin (10 mcg), Ampicillin/Sulbactam (10/10 mcg), Streptomycin (10 mcg), Choramphenicol (30 mcg), Colistin (10 mcg), Enrofloxacin (10 mcg), Amoxyclav (30 mcg), Vancomycin (30 mcg), Imipenem (10 mcg) and Doxycycline (30 mcg) were then placed in the plate. The plate was placed in refrigerator for 15 minutes for diffusion of antibiotics and then it was incubated aerobically at 37°C overnight. Zones of inhibition were measured and compared with zone size interpretative table furnished by the manufacturer and graded as either sensitive or resistant.

Results

Isolation and cultural identification of *E. coli*

The present research work was taken up with a view to ascertain the incidence, isolation, identification, biochemical characterization multiple and drug resistance pattern among the *E. coli* isolates from clinical cases of mastitis and diarrhoea in bovine. total 53 (30.64) isolates were identified as *E. coli* based on phenotypic identification from 173 samples from cases of bovine mastitis (n=128) and faecal samples (n=45) from diarrhoeic cows and buffaloes. After primary isolation on BHI, the Gram's stained smear revealed 53 Gram negative short rods and coccobacilli morphologically similar to *E. coli* under microscope.

Fig-1 Lactose fermenting pink coloured colonies of *E. coli* on MacConkey agar

Fig-2 *E. coli* colonies with characteristic greenish metallic sheen on eosin methylene blue (EMB) agar

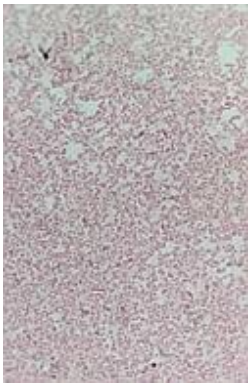

Fig-3 Negative short rods and coccobacilli morphologically similar to *E. coli* under microscope

Fig-4 Indole test

Fig-5 Methyl-Red (MR) test

Fig-6 Voges-Proskauer (VP) test

Fig-7 Citrate test

NOTE: P-Positive test, N-Negative test and C-Control

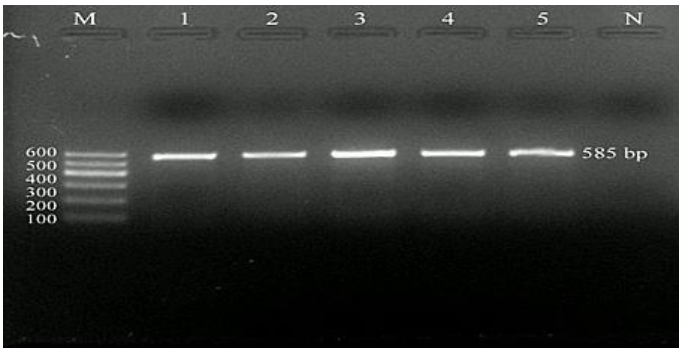

Fig-8 PCR amplification of 16S rRNA gene of *E. coli* isolates

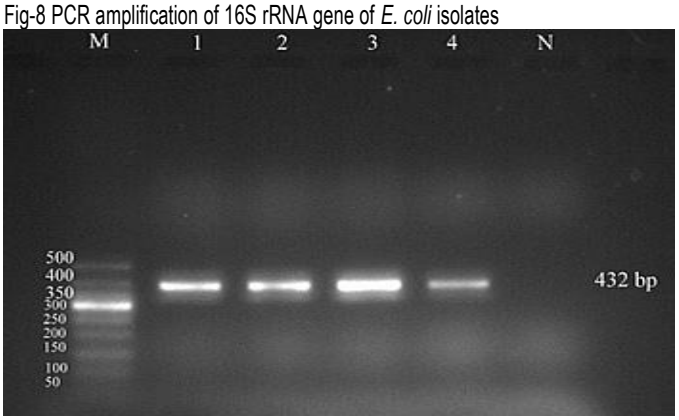

Fig-9 Agarose gel showing pcr amplified product (432 bp) for gene *ehly* in *E. coli* isolates

All the 53 isolates produced lactose fermenting pink coloured colonies on MacConkey agar medium and colonies with greenish metallic sheen on EMB agar medium. Based on colony characteristics, Gram's staining, growth on MacConkey agar and EMB agar, 53 presumptive *E. coli* isolates were recovered.

Biochemical identification of *E. coli* isolates

All the 53 presumptive *E. coli* isolates were further subjected to biochemical tests *viz*., indole, methyl red, Voges Proskauer and citrate tests. All the 53 isolates showed typical IMViC patterns of *E. coli viz*., indole and M.R positive and V.P and citrate negative.

Genotypic confirmation of *E. coli*

For Genotypic confirmation, all the 53 presumptive isolates of *E. coli* were subjected to PCR using species-specific 16S rRNA gene (*ECO-1*) amplification. All the 53 isolates successfully amplified 585 bp amplicon from the genomic DNA which confirmed them as a *E. coli*.

Virulence genes characterisation of *E. coli*

The analysis of virulence factors of *E. coli* isolates comprised the detection of Haemolysin (*ehly*) and Intimin (*eaeA*) genes by PCR.

Detection of *ehly* gene

Out of 53 isolates of *E. coli*, 13 (24.52 %) were found positive for *ehly* gene, in which amplicon size of 432 bp was detected. 32 (75.48%) isolates did not reveal any band indicating negative for *ehly* gene.

Detection of *eaeA* gene

All the 53 isolates did not reveal any band indicating negative for *eaeA* gene.

Antibiotic sensitivity patterns of *E. coli* isolates

Antibiotic sensitivity patterns of *E. coli* isolates were assessed on Muller Hinton media (Plate 4.6). All the 53 *E. coli* isolates were subjected to antibiotic susceptibility test against 18 different antibiotics which revealed different susceptibility patterns. Out of 53 isolates of *E. coli*, 42 (79.72%) isolates showed sensitivity to Chloramphenicol, 35 (66.36%) to Colistin, 31 (58.15%) to Cefepime, 30 (56.36%) to Amoxyclav, 29 (54.72%) to each Ceftazidime-clavulanic acid,

Table-5 *Sensitivity pattern of E. coli towards antibiotics alone and antibiotics with β-lactamase inhibitor*

Antibiotics	sensitivity (%)	Antibiotics with β-lactamase inhibitor	sensitivity (%)	Increase in sensitivity (%)
Ceftazidime	20.75	Ceftazidime-clavulanic acid	54.72	33.97
Ceftriaxone	32.08	Ceftriaxone-salbactam,	43.4	11.38
Cefotaxime	28.3	cefotaxime-clavulanic acid	45.28	16.98

Table-6 *Multidrug resistance patterns of E. coli isolates*

No. of Antibiotics	Antibiotics Resistance pattern	No. of solates	Total
	S,CEC,VA	1	
3	CAZ,VA,GEN	2	5
	CAZ,CEC,VA	1	
	CAZ,AMC,CPM	1	
	CAZ,AT,CIS,CL	1	
	CAZ,CTR,S,CIS	2	
	CAZ,CEC,VA,EX	1	
4	CAZ,CPM,CIS,VA	1	11
	CAZ.CEC,CIS,VA	3	
	CAZ,CTR,DO,VA	1	
	CTR,DO,CEC,VA	1	
	CAZ,IPM,CEC,EX	1	
	CAZ,CE,C,CIS,CL	1	
5	CAZ,CTR,CEC,CIS,VA	2	4
	CAZ,CTX,CEC,CIS,VA	1	
	CAZ,CPM,CIS,VA,GEN, EX	1	
6	CAZ,CPM,GEN,EX,CIS,VA	1	3
	CAZ,AMC,S,CIS,VA,CL	1	
	AMC,S ,IPM,CEC,CIS,VA,EX	1	
7	AMC,CPM,CAC,DO,CEC,CIS,VA	1	4
	CTR,CAC,DO,CEC,CIS,AS,CL	1	
	CAZ,AT,DO,CEC,CIS,CL,GEN	1	
	CAZ,CPM,CTR,AT,CAC,CTX,CEC,CIS	2	
8	CPM,AT,CAC,CTX,CEC,AS,CIS,VA	2	6
	CAZ,AMC,CPM,CTR, AT, CAC,S, YPM	1	
	CAZ,AT,IPM,DO,CEC,CIS,CL,GEN	1	
9	CAZ,CPM,CTR,AT,CAC,S,DO,CIS,CL	1	2
	CAZ,CPM,CTR,AT,CAC,CTX,CEC,CIS,CL	1	
	CAZ,AMP,IPM,DO,CTX,CEC,AS,C,VA,GEN	1	
10	CAZ,CTR,AT,CAC,S,IPM,CEC,C,VA,GEN	2	4
	CPM,CTR,S,DO,CTX,CEC,CIS,VA,CL,EX	1	
11	AMC,CPM,CTR,S,DO,CTX,CEC,CIS,VA,CL,EX	1	2
	CAZ,AMC,ATS,APM,CTX,CEC,CIS,VA,CL,GEN,EX	1	
12	CAZ,AMC,AT,S,IPM,CEC,CIS,CTX,VA,CL,GEN,EX	1	1
13	CAZ,AMC,CPM,CTR,CAC,S,IPM,DO,CTX,CEC,VA,GEN,EX	1	2
	CAZ,AMC,CTR,CAC,S,DO,CTX,CEC,CIS,C,VA,GEN,EX	1	
14	CAZ,AMC,CPM,CTR,AT,CAC,S,IPM,DO,CRX,CEC,CIS,C,VA	1	1
15	CAZ,AMC,CPM,CTR,CAC,S,IPM,DO,CTX,CEC,CIS,AS,VA, GEN, EX	1	2
	CAZ,AMC,CPM,CTR,AT,CAC,S,DO,CTX,CEC,CEC,CIS,C,VA, EX	1	
16	CAZ,AMC,CPM,CTR,AT,CAC,S,IPM,DO,CRX,CEC,CIS,A/S, GEN, EX,VA	1	1
17	CAZ,AMC,CPM,CTR,AT,CAC,S,IPM,DO,CTX,CEC,CIS,AS,VA,CL,GEN,EX	3	3

C:Chloramphenicol; CL:Colistin; CPM:Cefepime; AMC:Amoxyclav; CAC:Ceftazidime-Clavulanic acid; GEN:Gentamicin; IPM:Imipenem; AS:Ampicillin/sulbactam; EX:Enrofloxacin; AT:Aztreonam; CEC:Cefotaxime-Cavulanic acid; CIS:Ceftriaxone-Sulbactam; DO:Doxycycline; CTR:Ceftriaxone; CYX:cefotaxime; S:Streptomycin; CAZ:Ceftazidime; VA:Vancomycin

Gentamicin, Imipenem and Ampicillin/sulbactam, 28 (52.83%) to each Enrofloxacin and Aztreonam, 24 (45.28%) to Cefotaxime-clavulanic acid, 23 (43.40%) to Ceftriaxone-salbactam, 19 (35.85%) to Doxycycline, 17 (32.08%) to Ceftriaxone, 15 (28.30%) to Cefotaxime, 14 (27.79%) to Streptomycin, 11 (20.75%) to Ceftazidime and 7 (13.79%) to Vancomycin.

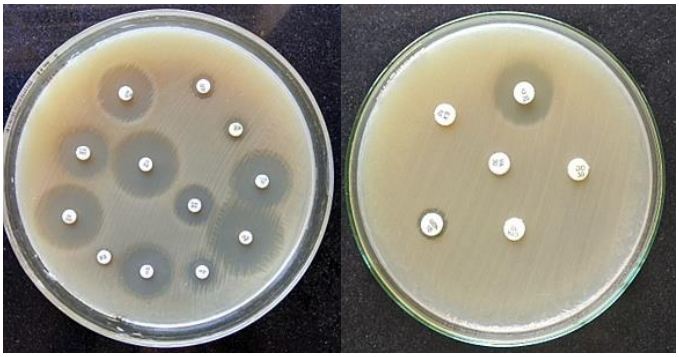

Fig-10 Plates showing antibiogram of *E. coli* isolate

Discussion

In this study, out of 128 milk samples from bovine mastitis evaluated for the presence of *E. coli*, 38 milk samples were found positive indicating incidence of 29.69 per cent. This is in agreement with the report by Yohannes (2018) [16] and Mohanty, *et al.,* (2013) [17] who found 25.00 and 21.00 per cent prevalence of *E. coli* from milk samples collected from bovine mastitis, respectively. In contrast to the current findings, Lye, *et al.,* (2013) [18] and Addo, *et al*., (2011) [19] reported lower incidence of 08.75% and 11.20% from Malaysia and Ghana, respectively. On the other hand, higher incidence of 69.00, 63.00 and 90.67 per cent was recorded by Fadaei (2014) [20] and Lubote, *et al.,* (2014) [21] who reported from Iran, Khartoum and Faecal samples (n=45) from diarrhoeic cows and buffaloes were processed for isolation of *E. coli*. 15 faecal samples were detected positive giving an incidence of 33.33 per cent which corroborates the findings of [22] and [23] who reported 24.00 and 37.00 per cent incidence of *E. coli* from calf diarrhoea, respectively.

All the 53 presumptive strains of *E. coli* isolates were subjected to Gram's staining and IMViC tests for identification. In Gram's stained culture smears under microscope, all the 53 isolates were observed as Gram negative with short rods and coccobacilli.

In the present study. Biochemical behaviour of the isolates revealed that they all were found positive for methyl red and indole production while negative for Voges-Proskauer test and failed to utilize citrate on Simmon's citrate agar. These results are in accordance with the bio-chemical characteristics of *E. coli* reported by Edwards and Ewing (1972) [24] and Barrow and Feltham (1993) [25].

For Genotypic confirmation, all the 53 presumptive isolates of *E. coli* were subjected to PCR using species-specific 16S rRNA gene (*ECO-1*) amplification. All the 53 isolates successfully amplified 585 bp amplicon from the genomic DNA which confirmed them as a *E. coli*. The same method of genotypic confirmation of *E. coli* using the similar primers was reported by Sohidullah, *et al.,* (2016) and Rahman, *et al.,* (2017) [26, 27].

The eae gene was not detected in the isolates tested. In line with the present study, [28, 29] reported incidence of eae gene in 01.80, 00.00 and 00.00 per cent *E. coli* isolates, respectively. The reason was all the isolates failed to amplify eae gene might be the fact that eae gene is mostly present in the clinical cases of bloody diarrhea [30] but in the present investigation none of the bloody diarrhea samples were processed.

Further, the *E. coli* isolates were screened for the presence of virulence gene *ehly*. Out of 53 *E. coli* isolate, *ehly* gene was present in 13 (24.52%) isolates which corroborates the findings of [31] and [12] who identified *ehly* gene in 14.29 and 26.88 per cent *E. coli* isolates, respectively using similar primers.

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.

Chloramphenicol showed maximum effectiveness of 79.25 per cent which corroborates the findings of [33] and [34] who observed 100.00, 76.00 and 80.00 per cent sensitivity to Chloramphenicol, respectively. In contrast to the present findings, 68.40 per cent resistance was recorded by [35].

According to its way of emergence, antimicrobial resistance can be either intrinsic, due to a lack of binding sites or other pharmacological characteristics, or acquired. The former can cause clinical problems but is not considered as a major public health issue while the acquired one has the potential for transmission to human beings and is of great concern to public health authorities [36]

In this study, higher rate of resistance *i.e*., 86.79, 79.25, 73.58, 71.70, 67.92 and 64.15 per cent was observed towards Vancomycin, Ceftazidime, Streptomycin, Cefotaxime, Ceftriaxone and Doxycycline, respectively. The result of the present study was somewhat supported by the findings of others with the respect to antibiotic sensitivity profile of *E. coli* isolates. In accordance with present finding, [37] reported that Ampicillin, Cefotaxime, Ceftazidime and Cefuroxime showed 100.00 per cent resistance towards *E. coli* strains isolated from cattle. [38] reported that *E. coli* isolates of buffalo mastitis showed 100.00 per cent resistance for Amikacin, Amoxycillin-sulbactam, Ampicillin, Cefotaxime, Ceftriaxone-sulbactam. [39] tested 231 *E. coli* isolates from bovine mastitic milk and reported a high antimicrobial drug resistant especially for Amoxicillin + Clavulanic acid (85.70%), Ceftriaxone (82.20%) and Cotrimoxazole (68.80%). [40] also studied the prevalence of drug resistant *E. coli* isolates of mastitis in Tamil Nadu and reported resistance to Amoxicillin (53.00%), Oxytetracycline (58.00%), Penicillin-G (60.50%), Oxacillin (56.30%), Gentamicin (43.70%), Enrofloxcain (43.70%), Amoxicillin + Sulbactam (49.60%) and Ceftriaxone (13.40%).

The antibiotic group of choice in case of bovine mastitis caused by *E. coli* is β-lactam antibiotic group which is used broadly in veterinary medicine. Unfortunately, *E. coli* bacteria have developed resistance against most of β-lactam antibiotics. In case of beta-lactam antibiotics, higher resistance rate of 79.50, 71.50, 68.15, 47.00, 45.22 and 41.86 per cent was observed towards Ceftazidime (30 mcg), Cefotaxime (30 mcg), Ceftriaxone (30 mcg), Aztreonam (30 mcg), Carbapenem (10 mcg) and Cefepime (30 mcg), respectively. Bacterial resistance to β-lactams, popular antibiotics due to their proven safety and efficiency, is increasing at an alarming rate. This resistance is mainly achieved through β-lactamases that can hydrolyse most β-lactam antibiotics including the third and fourth generation ESCs and monobactams [41]. ESBLs are predominantly produced in gram negative bacteria, particularly in *E. coli*, and are considered a key mechanism conferring resistance to Cephalosporins [42].

It is quite interesting to note that when the isolates were tested against antibiotics with β-lactamase inhibitor, they showed maximum efficacy against *E. coli* isolates, however these isolates showed resistance against same antibiotics alone without β-lactamase inhibitor. In the present study four antibiotics *viz*., Ceftazidime, Ceftriaxone, Cefotaxime and Ceftazidime were used alone as well as with β-lactamase inhibitor *viz*., Sulbactam and clavulinic acid. The details of sensitivity pattern of *E. coli* towards antibiotics alone and antibiotics with β-lactamase inhibitor is depicted in [Table-5]. This may be because β-lactamase inhibitor *viz*., Sulbactam and clavulinic acid blocks the β-lactamase enzyme which breaks down β-lactam ring of antibiotic and thereby allows antibiotic to attack and kill the bacteria.

Magiorakos, *et al*., (2012) [43] defined MDR as non-susceptibility to at least one agent in three or more antimicrobial categories. Based on the finding of the present study, total 96.23% isolates of bovine clinical samples were categorized as MDR (resistant to ≥3 to 17 antimicrobial categories). Antibiogram of 53 *E. coli* isolates revealed that 09.43, 20.75, 07.53, 05.66, 07.55, 11.32, 03.77, 07.55, 03.77, 01.88, 03.77, 01.88, 03.77, 01.88 and 05.67 per cent were resistant to 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17 antibiotics categories, respectively. The category wise details of antibiotic resistance pattern are set out in [Table-6]. The prevalence of MDR *E. coli* in bovine clinical samples (96.23%) was higher than earlier reports of [44] who recorded the prevalence of 70.69, 50.00 and 100 per cent MDR *E. coli* from urine, milk and uterine discharge samples, respectively. [45] characterized multidrug-resistant (MDR) *E. coli* isolates collected from Serbia from bovine clinical mastitis cases and diseased pigs, during the years 2013–2014, and reported prevalence of 45.83 per cent isolates as MDR.

Conclusion

E. coli isolated from clinical bovine mastitis and diarrheagenic *E. coli* are typical commensal. Almost all the isolates are multidrug resistant which might be associated with the overuse of respective antibiotics to control mastitis or other disease condition of the affected animals. This study demonstrated that *E. coli* were susceptible to Chloramphenicol, Colistin, Cefepime and Amoxyclav but resistant to Streptomycin, Ceftazidime and Vancomycin. The findings of this research work would certainly help to select the proper antibiotics against diarrhoea and mastitis in cattle to overcome the multi-drug resistant problem of the bacteria. Occurrence of multidrug resistant *E. coli* is alarming and indicates a potential risk of transferring multidrug resistant *E. coli* and resistance to human, animal and nature through the contamination milk or milk products.

Application of research: *E. coli* one of the major contributor for the cause of mastitis and diarrhea. By identifying virulence gene and antibiotic sensitivity pattern of *E. coli*. helpful in understanding development of disease process and treating diseases in animals.

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Study area / Sample Collection: Banaskantha, Mahesana, Sabarkantha and Patan districts of Gujarat

Cultivar / Variety / Breed name: Nil

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