Original Research Article https://doi.org/10.20546/ijcmas.2019.806.238

Isolation, Identification, Molecular Characterization and Antibiogram of *E. coli* Isolates from Neonatal Calves

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A B S T R A C T

Neonatal calf diarrhoea (NCD) is a major threat to dairy industries. It is a multi-etiology disease with *E. coli* as the predominant cause. Emergence of antimicrobial resistance is of utmost concern because it has rendered the present day antibiotics less effective. The present study aims for isolation, molecular characterization and antimicrobial resistant profiling of *E. coli* from neonatal calves. One hundred two fecal samples were collected from neonatal calves for isolation of *E. coli*. All the isolates showed positive result on biochemical analysis. But only 78.43% (80/102) of them were found positive for *usp*A (universal stress protein) gene. The nucleotide sequences flanking the gene encoding the *usp*A and was found to be highly specific for *E. coli*. The antibiogram of *E. coli* isolates by Kirby Bauer disc diffusion method revealed highest resistance against kanamycin (97%) and lowest against gentamicin (5.86%). Eighty three percent (85/102) isolates were MDR. 39 (38.24%) isolates were phenotypic ESBL producers (by combination disc diffusion method) and 22 (21.57%) isolates were phenotypic carbapenem resistant (against Ertapenem, Meropenem, Imipenem, Doripenam) Hence, surveillance based study and search for an alternative to antibiotics is needed to address the global issue of the antimicrobial resistance.

Introduction

Sustainable livestock production depends on the successful raising of healthy neonates (Muktar *et al.,* 2015). Neonatal diseases and mortality among the cattle and buffalo calves are the major cause of economic losses in livestock production (Singh *et al.,* 2009). It has been estimated that 75% of early calf mortality in dairy herds is due to acute diarrhea during the pre-weaning period (Blowey, 1990). Diarrhea in neonatal calves is a complex, multi-factorial and most dynamic disease (Lorenz *et al.,* 2011). Typical calf diarrhea outbreaks often involve mixed infections of virus, bacteria and other agents which are a potent source of zoonosis. The most important group of bacteria causing calf diarrhea are *E. coli*, *Salmonella* and *Clostridium perfringens.* *E. coli* is the predominant aerobic organism in the normal intestinal microbiota of mammals and plays

an important role in host metabolism, immunology and nutrition (Tenaillon *et al.,* 2010). It enhances digestion process and helps in nutrient assimilation. But in debilitated or immunosuppressed host or when gastrointestinal barriers are violated, even normal non-pathogenic strains of *E. coli* can cause infection. Pathogenic *E. coli* is one of the most common groups of bacteria causing diarrhea and extra intestinal infections in both animals and human (Levine, 1987). *Escherichia coli* has six *usp* genes namely A, C, D, E, F and G, and the *usp*A gene is important for the survival of *E. coli* during cellular growth, adhesion and motility (Nachin *et al.,* 2005). Synthesis of *usp* protein is induced in response to stress such as starvation, heat shock, presence of toxins or osmotic pressure (Nyström and Neidhardt, 1992), however, cold shock doesn't induce synthesis of *usp*A (Nyström and Neidhardt, 1993).

Rise of Multi-Drug Resistant (MDR) microbes in early 1960s was a major setback for the antibiotics industry. These MDRs are taking us back to the pre-antibiotic era where minor infections and injury was fatal. *E. coli* serves as a sentinel organism for antimicrobial resistance development in various animals (Allen *et al.,* 2010).

It is an enteric commensal, can be a pathogen, and easily acquires resistance. It can play a role in transfer of resistance to other species or pathogens (Aarestrup *et al.,* 1999; Stobberingh and Bogaard *et al.,* 2000; Allen *et al.,* 2010; Ashbolt *et al.,* 2013). This inevitable process of development antimicrobial resistance could be slowed down only by judicious use of appropriate antibiotics. Hence, surveillance based study, antimicrobial resistance profiling and search for an alternative to antibiotics is the need of hour to address the global issue of the antimicrobial resistance. In this study,

isolation, biochemical analysis, molecular characterization by *usp*A gene and antibiotic resistance profiling of 102 *E. coli* isolates from neonatal calves was done.

Material and Methods

Sample collection

Fecal samples of neonatal calves of cattle and buffalo were collected from Cattle and Buffalo Farm, Livestock Product Management section, IVRI, Izatnagar. Basic information about the dairy farm and management was taken. A total of 102 fecal samples including 14 diarrhoeic samples were collected from calves of cattle (Vrindavani and Tharparkar) and buffalo (Murrah) of below 3 months of age. Sterile fecal swabs (Hi-media, India) were used for sample collection. All the samples were transported immediately to the laboratory under cold chain and were processed for isolation of *E. coli* using standard microbiological techniques.

Bacterial isolation

Enrichment media

For isolation of *E.coli,* a loopful of fecal sample was enriched in BPW (Buffered Peptone Water), and incubated at 37 °C for 16-18 hours.

Selective media

Enriched culture was inoculated into MacConkey's agar and incubated at 37 °C for 18–24 hours.

Differential media

Pink colour colonies on MacConkey's agar were selectively picked and streaked aseptically onto Eosin Methylene Blue (EMB)

agar and incubated at 37 °C for 18–24 hours. The isolates showing atypically colonies were once again streaked on fresh EMB plates.

Identification of *E. coli*

Gram's staining

Partially identified cultures were suspended in normal saline and smeared over a slide. The smears were allowed to air dry followed by heat fixing and Gram's staining.

Molecular characterization

DNA extraction

The bacteria grown for 18 hr in LB broth were pelleted in 1.5 ml micro centrifuge tube by centrifugation for 10 min at 5000 x *g* (7500 rpm). The genomic DNA of the isolates was extracted by using QIAamp DNA Mini Kit (Qiagen, USA) following the manufacturer's protocol.

PCR confirmation

All the biochemically confirmed isolates where subjected to PCR for the presence of *usp*A gene (884bp). Species specific primers (F-5'-CCGATACGCTGCCAATCAGT-3' and R-5'-ACGCAGACCGTAAGGGCCA GAT-3') were used to amplify the *usp*A genes of *E. coli* (Osek, 2001; Rajput *et al.,* 2014) which was procured from Eurofins Genomics India Pvt. Ltd., Bengaluru. PCR reaction was performed in a volume of 25 µl containing 1.5 units of *Taq* DNA polymerase, 1.5 mM MgCl$_2$, 200 µM of each deoxynucleoside triphosphate, 10 pmol of each primer, 50 ng of template DNA and nuclease free water up to 25 µl. PCR conditions are mentioned in table1 (Mishra *et. al*. 2017). The amplified PCR products (10 µl aliquots) were analyzed by electrophoresis in 1.5% agarose gels in tris acetate EDTA buffer at 100 V. The gels were stained with ethidium bromide and viewed under ultraviolet light using a commercial documentation system (Alpha Innotech, USA).

Biochemical characterization

All Gram negative culture were subjected to biochemical characterization by using a HilMViC kit which consists of combination of 12 tests; four conventional biochemical tests and eight carbohydrate utilization tests. Kit contained sterile media for indole, methyl red, Voges Proskauer's, citrate utilization tests and 8 different carbohydrates i.e. glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose and sucrose.

The standard test procedure mentioned in the kit was followed. The results were interpreted as per the standards given in the interpretation chart provided by the manufacture of the kit. *E. coli* reference strains available at National *Salmonella* Centre, IVRI were used as positive control for biochemical identification.

Antimicrobial resistance profiling

Antibiogram

The detailed antibiogram of *E. coli* isolates was determined by against 16 antibiotics belonging to 8 different classes by Kirby Bauer disc diffusion method (CLSI, 2015). Disc used were Aminoglycosides (streptomycin 10µg, gentamicin 10µg, kanamycin 30µg, amikacin 30g) Polymyxin (colistin 10µg), Cephalosporins–3rd generation (cefotaxime 30 µg, ceftazidime 30µg), Fluoroquinolone (enrofloxacin 10µg, ciprofloxacin 5µg, ofloxacin 5µg), Penicillins (ampicillin 10 µg), Tetracyclines (tetracyclines 30 µg), Sulphonamides (cotrimoxazole 25 µg, sulphadiazine 100 µg), nitrofurantoin 30µg and trimethoprim.

The *E. coli* isolates were grown in 5 ml LB broth (BD, BBL Difco, USA) at 37°C for 8 hr to develop a turbidity of 0.5 McFarland standards. Uniform bacterial lawn was prepared on sterile Mueller Hinton Agar (MHA BD, BBL Difco, USA) plates surface with the help of cotton swab using aseptic technique. The antibiotic discs were placed on MHA with adequate spacing between each other with sterilized forceps. The discs were gently pressed to ensure full contact with the medium. The plates were then incubated overnight at 37°C. Next day, the total diameter of the zone of inhibition was recorded in mm. The result was interpreted as per standard guidelines (CLSI, 2015).

Phenotypic detection of carbapenam resistant *E. coli*

Phenotypic carbapenem resistant isolates were detected by Kirby Bauer disc diffusion method (CLSI, 2015) using 10μg disc of ertapenem, meropenem, imipenem and doripenem.

Phenotypic detection of ESBL producing *E. coli* by combination disc method

All the *E. coli* isolates were inoculated in Luria Bertani (LB) broth for overnight incubation at 37°C. The bacterial inoculums were standardized by measuring the optical density (O.D) at 600 nm wavelength to 0.5 McFarland standards. The bacterial lawn was prepared on MHA plates and subsequently discs of ceftazidime (30 μg), and cefotaxime (30 μg) were diagonally placed with the corresponding discs of ceftazidime + clavulanic acid (30/10 μg) and cefotaxime + clavulanic acid (30/10 μg), respectively. An increase of 5 mm or more in the zone of inhibition of disc containing the antibiotic along with clavulanic acid than the disc containing the antibiotic alone was indicative of ESBL producer (fig.1).

Results and Discussion

Isolation and identification of *E. coli*

Out of 102 fecal samples, 14 were diarrhoeic samples and all were positive for *E. coli.* The pink colour colony on MAC Agar indicating the lactose fermenter (fig. 2) when further streaked on EMB showed characteristics green metallic sheen (fig.3) which was further characterized by biochemical kit (fig. 4). *E. coli* is Indole and MR positive and VP and Citrate negative, Carbohydrate utilization test result was glucose +, adonitol−, arabinose+, lactose+, sorbitol+, mannitol+, rhamnose-V, sucrose −V (variable). On the basis of microbiological and biochemical analysis, all the fecal samples were found positive for the *E. coli* i.e. isolation rate was 100%.

Molecular characterization

Out of 102 *E. coli* isolates 80 were positive for *usp*A gene (fig.5). *E. coli* isolated from diarrhoeic samples was all positive for *usp*A gene. There was difference of 21.57% in phenotypic and genotypic expression of *E. coli* isolates.

Antibacterial drug resistance profiling of the *E. coli* isolates

Antibiogram of *E. coli* isolates

Antibiotic susceptibility pattern of the 102 *E. coli* isolates were determined by Kirby Bauer disc diffusion method against 16 different antibiotics (fig.6). The overall resistance (resistant + intermediate) was maximum against kanamycin (97%) followed by tetracycline (68.6%), ceftazidime (63.7%), Nitroflurantoin (52%) ampicilin (51%), trimethoprime (47%), streptomycin (44.1%) co-trimoxazole and sulphadiazine (42%). Maximum sensitivity was observed against gentamicin (94.14%) and colistin (93.1%),

followed by ciprofloxacin (81.4%), ofloxacin (77.5%) and amikacin (69.6%) (Table 2; Fig. 7). There was wide spread multidrug resistance among the *E. coli* isolates, 83.33% (85/102) of isolates were multi-drug resistant i.e. resistant against 3 or more antibiotics.

ESBL producing *E. coli*

A total of 39 (38.24%) isolates were phenotypic ESBL producer. Prevalence of ESBL among healthy calves was 32.95% (29/88), whereas among diarrheic calves was 71.43% (10/14).

Carbapenem resistant *E. coli*

Out of the 102 isolates, 22 (21.57%) were found to be carbapenem resistant. Carbapenem resistance was 21.6 % (19/88) from healthy calves, whereas from that of diarrheic calves it was just 13.64 % (3/22).

Among the carbapenem, maximum resistance was seen against ertapenem (14/22), and meropenem, imipenem and doripenem resistant isolates were two each (2/22).

Neonatal calf mortality

Neonatal diseases and mortality among cattle and buffalo is the major cause of economic losses in the livestock sector (Singh *et al.,* 2009). High morbidity and mortality during neonatal period is primarily due to diarrhea (De la Fuente *et al.,* 1999; Svensson *et al.,* 2006). *E. coli* is considered as the most common bacterial cause of neonatal diarrhea. In the present study, all the 102 fecal samples (88 healthy and 14 diarrhoeic samples) were positive for *E. coli*. A similar finding was reported by the Ibrahim (1995). Hemashenpagam *et al.,* (2009) in India reported 75% prevalence, whereas Malik *et al.,* (2012) and Joon and Kaura (1993) reported much less prevalence (37% and 23%). The differences in the prevalence rates of *E. coli* among diarrheic calves may be attributed to the geographical locations of the farm, management practice and hygienic measures (El-Seedy *et al.,* 2016). Therefore, it is imperative to study the prevalence of enteropathogens and related risk factors which can modulate the occurrence of diarrhoea among calves.
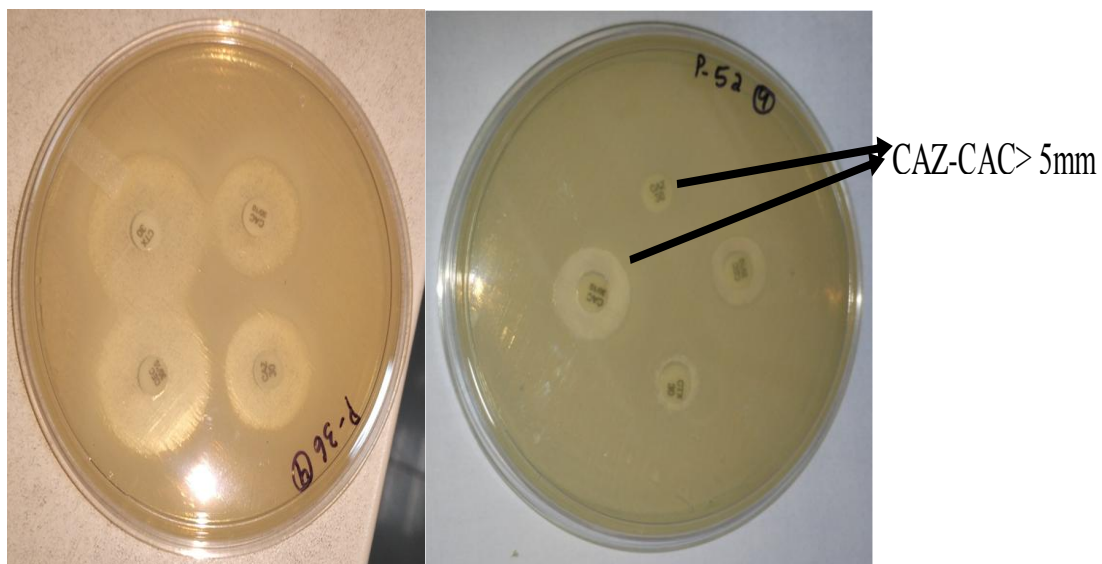

CAZ-CAC> 5mm

Fig 1: Combined Disc Diffusion Method for ESBL producing *E. coli*

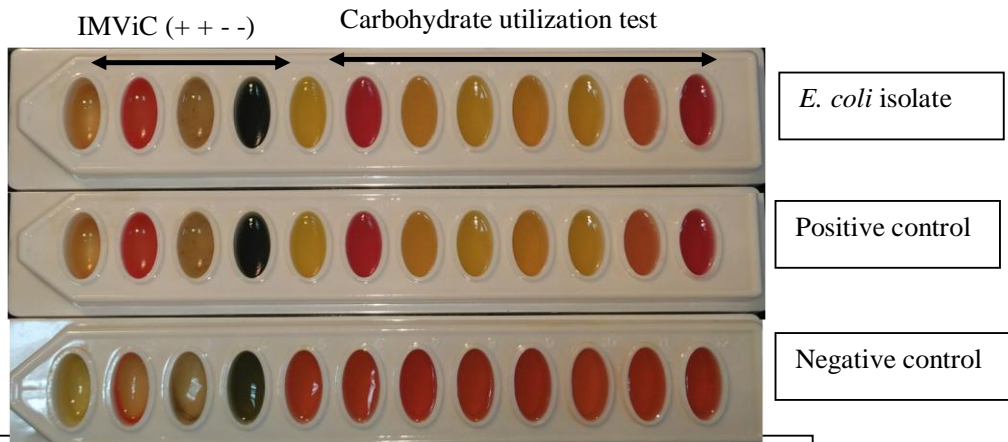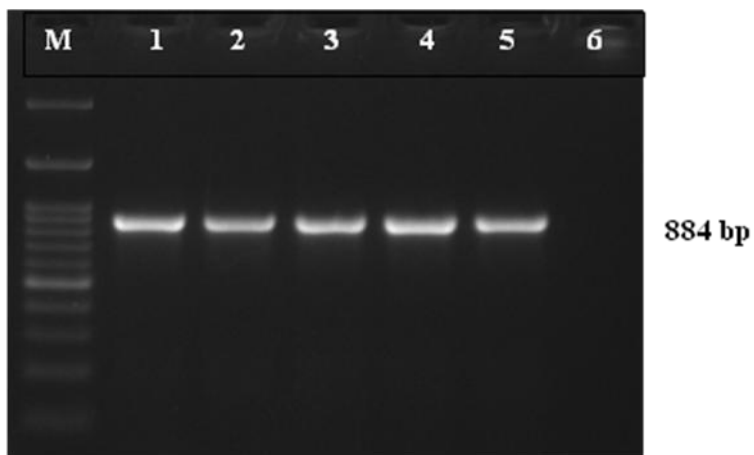| Fig.2 Pink colour colonies of *E.coli* on MAC	Fig.3 Green metallic sheen of *E.coli* on EMB Agar

Fig 4: Biochemical Test of *E .coli* using HilMViC kit

M: 100 bp ladder, Lane 1 to 5: samples positive for
*usp*A gene (884bp); Lane 6: negative control
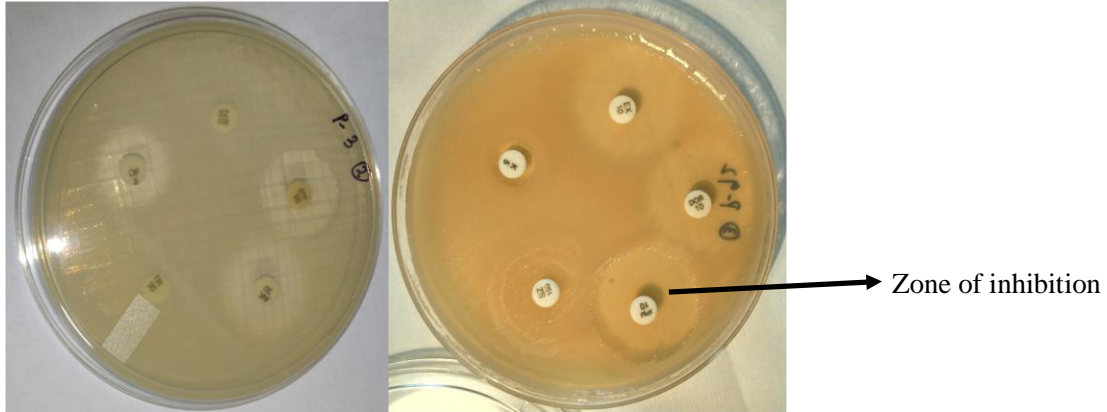**Fig. 5: PCR Confirmation of *usp*A gene**
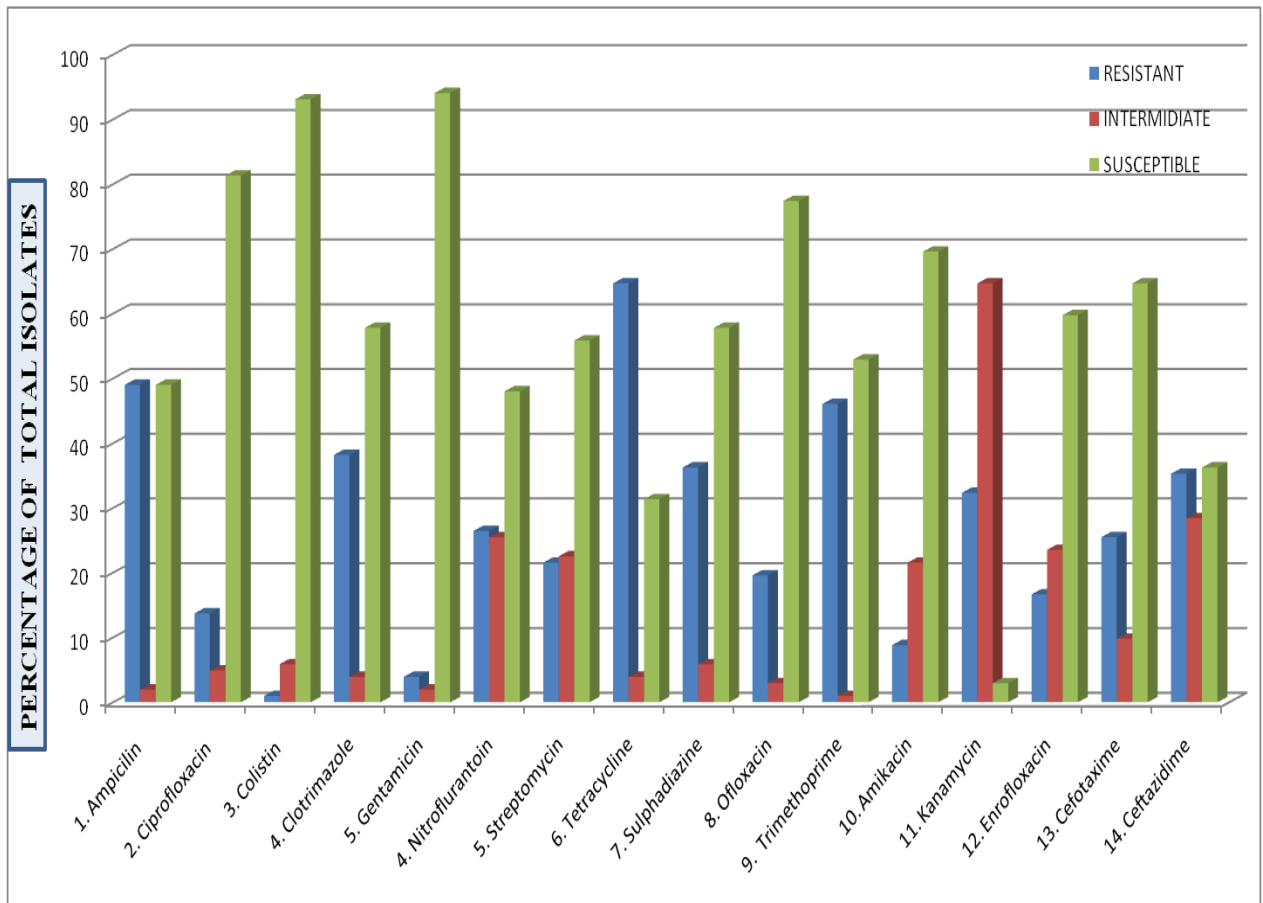
Fig. 6: Kirby Bauer disc diffusion method

Zone of inhibition

Fig. 7 : Antibiogram of *E. coli*

Table.1 PCR conditions for detection of *usp*A gene

PCR steps	Temperature	Duration	Cycles
Initial denaturation	95°C	2min	1
Denaturation	94°C	30sec	30
Annealing	58°C	60sec	30
Extension	72°C	60sec	30
Elongation	72°C	5min	1
Cooling	4°C	Infinite	

Table.2 Antibiotic susceptibility profile of *E. coli* isolates

	Percentage of isolates		
	Resistant	Intermediate	Susceptible
Ampicilin (AMP-10)	49.02	1.96	49.02
Ciprofloxacin (CIP5)	13.72	4.9	81.34
Colistin (CL-10)	0.98	5.88	93.1
Clotrimazole (COT25)	38.22	3.92	57.82
Gentamicin (GEN10)	3.92	1.96	94.08
Nitroflurantoin (NIT10)	26.46	25.48	48.02
Streptomycin (S10)	21.56	22.54	55.86
Tetracycline (TE30)	64.68	3.92	31.36
Sulphadiazine (SZ100)	36.26	5.88	57.82
Ofloxacin (OF5)	19.6	2.94	77.42
Trimethoprime (TR)	46.06	0.98	52.92
Amikacin (AK30)	8.82	21.56	69.58
Kanamycin (K30)	32.34	64.68	2.94
Enrofloxacin (EX10)	16.66	23.52	59.78
Cefotaxime (CTX30)	25.48	9.8	64.68
Ceftazidime (CAZ30)	35.28	28.42	36.26

Molecular confirmation by *usp*A gene

In present study 78.43% of the total isolates harvested *usp*A gene which is a universal stress protein specific for *E. coli.* There was difference of 21.57% in phenotypic and genotypic expression of *E. coli* isolates. *Usp*A is essential for the bacteria to cope with the cellular defence mechanism and oxidative stress (Nachin *et al.,* 2005; Siegle, 2005). It can also be targeted for many immunological assays, and can be included as a candidate for multiple subunit vaccines. In a study

Godambe *et al.,* (2017) stated that two molecular markers (*uid*A and flanking region of *usp*A) along with microbiological method were specific for the *E. coli*, in which 77% of the isolates were positive for both the genes but, 22% of the *E. coli* isolates were positive for any one of the two genes. In contrast, Molina *et al.,* (2015) stated that yaiO and lacZ3 primers are unique to *E. coli*, unfortunately these were not tested in field. Although, molecular confirmation is better than phenotypic detection, there are possibilities that molecular detection based on

one marker gene may give false negative results. PCR based confirmation of typical and atypical colonies obtained on selective plate for *E. coli* would give much accurate result.

Antimicrobial resistance

Extensive use of antibiotics in animal husbandry and veterinary practices at sub-therapeutic levels for growth promotion, prophylaxis and treatment had led to the development of antibiotic resistance in bacteria (Wileman *et al.,* 2009). Antimicrobial resistance is an increasing global threat to both human and animal health (Tadesse *et al.,* 2012; WHO, 2012). In present study, Antibiogram pattern was recorded against 16 different antibiotics revealed 83.33% (85/102) were MDR. The overall resistance was maximum against kanamycin (97%) followed by tetracycline (68.6%), ceftazidime (63.7%), Nitroflurantoin (52%) ampicilin (51%), trimethoprime (47%), streptomycin (44.1%) co-trimoxazole and sulphadiazine (42%). Maximum sensitivity was observed against gentamicin (94.14%) and colistin (93.1%), followed by ciprofloxacin (81.4%), ofloxacin (77.5%) and amikacin (69.6%). A study on antimicrobial susceptibility of 96 *E. coli* isolates recovered from diarrheic calves of less than 3 months of age revealed high resistance against all antibiotics except for marbofloxacin, spectinomycin and neomycin (El seedy *et al.,* 2016). Khachatryan *et al.,* (2004) hypothesized that neonate-adapted bacteria were responsible for the high frequencies of resistant *E. coli.* Another study conducted in Uttar Pradesh, India by Malik *et al.,* (2012) reported higher sensitivity to amikacin (87.80%) and gentamicin (51.21%) and antimicrobial resistant profile was similar to our findings. However, 100% resistance were recorded against ampicillin, co-trimoxazole, erythromycin, and tetracycline. Shahrani *et*

al., (2014) reported high resistance to penicillin (100%), streptomycin (98.25%) and tetracycline (98.09%). Ansari *et al.,* (2014) reported similar type of findings where 100% resistant was observed against amoxycillin and tetracycline, 80% to Carbinicillin, 60% to cefotaxime and 20% to bacitracin and erythromycin. Ibrahim *et al.,* (2016) observed high resistance against ampicillin (56.3%), and low resistance against imipenem (1.6%).

There is a worldwide increase in the extended-spectrum β-lactamases (ESBLs) in Enterobacteriacae, particularly *E. coli.* The use of third generation cephalosporin's (cefotaxime, ceftazidime, ceftriaxone) in human medicine is generally believed to have been a major selective force in the emergence of ESBLs. In the present study, 39 (38.24%) isolates were ESBL producers (32.95% from healthy and 71.43% from diarrheic calves), and 22 (21.57%) were carbapenem resistant; 21.6 % (19/88) from healthy calves and 13.64 % (3/22) from diarrheic calves. Among the carbapenem, maximum resistance was seen against ertapenem (14/22), and remaining meropenem, imipenem and doripenem resistant isolates were two each (2/22).

One possible reason for such a wide spread resistance may be intrinsic or acquired through spontaneous mutations (de novo), or may occur due to horizontal gene transfer from donor bacteria, phages, or free DNA (Dodd, 2012). Due to horizontal gene transfer even nonpathogenic microbial species that harbour resistant genes serve as an ecological reservoir for pathogenic bacteria (Salyers and Shoemaker, 2006). These mechanisms include the uptake of naked DNA and mobile genetic elements such as plasmids, transposons, integrons, gene cassettes, and bacteriopahges (Nwosu, 2001).

It can be concluded from the present study that; both diarrhoeic and normal calves

harvested MDR *E. coli* which was around 83% (85/102) isolates. 80/102 isolates were positive for specific *usp*A gene. 39 (38.24%) isolates were phenotypic ESBL producers and 22 (21.57%) isolates were phenotypic carbapenem resistant. The increasing trends of multiple drug resistance among *E coli* isolates from neonatal calves are of utmost concern. The large number of calves dies during pre-weaning age causing heavy loss to livestock production and in turn to economy. Hence, surveillance based study, antimicrobial resistance profiling and search for an alternative to antibiotics is the need of hour to address the global issue of the antimicrobial resistance.

Acknowledgments

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