

Original Research Article

Prevalence of Multi-Drug Resistant (MDR) *Escherichia coli* in bovine clinical samples

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ABSTRACT

The present study was undertaken to assess the prevalence of *E. coli* in bovine clinical cases and examine their antibiotic sensitivity profile. A total of 200 bovine clinical samples including urine and milk and uterine discharge were subjected to isolation of *E. coli* followed by species specific PCR. The confirmed isolated were further subjected to antibiotic sensitivity test. The biochemical and molecular method showed a prevalence of 35.50% *E. coli* in bovine clinical samples with 45.67%, 19.05% and 10.0% in urine, milk and uterine discharge samples, respectively. Based on the finding of present study, a total of 66.20% isolates of bovine clinical samples were categorized as MDR (resistant to ≥ 3 to 6 antimicrobial categories). The prevalence of MDR *E. coli* in bovine clinical samples was 70.69%, 50.00% and 100% in urine, milk and uterine discharge samples, respectively. Out of 58 *E. coli* isolates from urine 22.41%, 18.96%, 18.96% and 8.6% were resistant to 3, 4, 5 and 6 antimicrobial categories, respectively. In 12 *E. coli* isolates from clinical milk samples 33.33%, 8.33% and 8.33% were resistant to 3, 4 and 5 antimicrobial categories, respectively. A single isolate from uterine discharge showed a resistant with 5 antimicrobial categories. The findings of the present study provides an insights into the epidemiological characteristics of clinical *E. coli* isolates from bovine and suggest the need for the prudent use of antimicrobial agents in husbandry and livestock clinics along with the urgent need to establish a national antibiotic resistance monitoring program.

Keywords

E. coli,
MDR *E. coli*,
Mastitis,
Antibiotic
resistance,
Antibiogram,
Bovine

Introduction

Escherichia coli, the head of gamma-proteobacterium in the family *Enterobacteriaceae*, found naturally as commensal in the intestinal tract of humans and many other animal species. They are also known to cause intestinal and extra-intestinal diseases (Kaper *et al.*, 2004; Fairbrother *et al.*, 2006). An *E. coli* subset is capable of causing enteric / diarrhoeal disease, and a different subset cause extra-

intestinal disease, including mastitis and urinary tract infections. Mastitis causes great economic losses to the dairy mainly due to reduction of milk yield (Seegers *et al.*, 2003). *E. coli* and *Klebsiella* are coliforms that can cause mastitis (Schukken *et al.*, 2011). The endotoxins of Gram negative bacteria lead to severe inflammatory response upon intra mammary infection. *E. coli* most frequently induce acute form of

clinical mastitis, which progresses rapidly and sometimes proved to be fatal (Sandholm & Pyorala, 1995). *E. coli* can also cause subclinical mastitis, although less frequently (Dogan *et al.*, 2005). Wide variation is recorded in frequency of *E. coli* isolated from mastitis cases depending on herd and country. In human, *E. coli* is seen as a major cause of urinary tract infections. However, reports on their role in urinary tract infection in animals are only meagre. Although, several pathogens have been involved in uterine infections, *E. coli* is one of the most frequently isolated organisms from infected uterine lumen. *E. coli* infections are also believed to predispose subsequent infections with other bacteria or viruses. Antimicrobials are used substantially for preventing and controlling diseases, improving growth performances and increasing feed efficiency in food producing animals (CDC, 2005). This liberal indiscriminate use of antibiotics results in emergence of resistant micro-organisms (Philips *et al.*, 2004). Antibiotics provide selection pressure for emergence and dissemination of antibiotic-resistant microorganisms (Levy, 1982; Witte, 1998). Recently, the incidence of multiple drugs resistance in *E. coli* has been reported to be on rise (Van den Bogaard, 1997; Witte, 1998; Khan *et al.*, 2005; Sharada *et al.*, 2010). Thus, keeping in the view the reports of emergence of multi drug resistant *E. coli*, the present study was designed with an objective to isolate and identify *E. coli* from clinical samples of bovine and generate antibiotic resistance profile of isolates.

Materials and Methods

Samples

A total of two hundred (200) samples (urine 127, milk 63 and uterine discharge 10) were collected from bovine clinical cases

presented at TVCC, Bihar Veterinary College, Patna during period of August 2016 to May 2017. Approx. one ml of different samples was collected aseptically in a sterile sample container.

Enrichment and Isolation on selective media

Approximately 1 ml of samples were inoculated with 10 ml of MacConkey broth and incubated for 24 hr at 44°C (Feng *et al.*, 2016). A loop full of MacConkey broth grown overnight and showing turbidity was streaked on the EMB agar and incubated at 37°C for 24 hr. Characteristic colonies of the *E. coli* on EMB agar i.e., purple with black centre and green metallic sheen colonies were selected for further studies.

Biochemical characterization of presumptive *E. coli* isolates

The colonies of presumptive *E. coli* on EMB agar were confirmed by biochemical test using HiIMViC Biochemical test kit (Himedia, India). A single presumptive colony from each clinical samples were grown in 5 ml nutrient broth for 4-6 h at 37°C and 0.5 µl of culture was inoculated on each well of test kit and incubated for 24 h at 37°C. The results were interpreted after addition of reagents in wells as per manufacturer guidelines (Himedia, India).

Molecular characterization of presumptive *E. coli* isolates

To confirm *E. coli* isolates with PCR species specific *16SrRNA* gene was amplified as per the method described by Sabat *et al.* (2000) with minor modification. The PCR was performed using the bacterial lysate as template DNA prepared by snap chill method (Swetha *et al.*, 2015). Amplification reaction was performed in 25 µl reaction

volume each containing 2.5 µl 10X PCR amplification buffer [500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM MgCl₂], 2.5 µl of dNTP (2.0 mM), 2.0 µl (10 pmol) of forward and reverse primers of 16SrRNA gene, 0.2 µl Taq DNA polymerase (5 unit/µl), 5.0 µl of bacterial lysate and nucleus free water upto 25 µl was used to amplify 16SrRNA gene of 544 bp. The PCR programme included initial denaturation at 94°C for 3 min followed by 40 cycles of denaturation (94°C for 30 sec), annealing (72°C for 45 sec) and extension (72°C for 45 sec) with final extension at 72°C for 10 min. To estimate the length of fragments, a 100-bp plus DNA ladder was run on each gel and the amplicons were observed and documented under gel documentation system. Primers used in the study were custom synthesized from Xcelris (India).

Antibiotic susceptibility study

The antibiotic susceptibility pattern was performed by disc diffusion method (Wayne, 2002). For this first of all, the test isolate was inoculated overnight in nutrient broth at 37°C. About 100 µl of the growth culture was spread on Mueller-Hilton agar plates with sterile L- spreader and antibiotic disc of antibiotics namely- ampicillin/sulbactam (10/10 µg) ciprofloxacin (5µg), amoxiclav (30µg), ofloxacin (5µg), amikacin (30µg), cefotaxime/ clavulanic acid (30/10µg), cefotaxime (30µg), ceftriaxone (30µg), chloramphenicol (30µg), penicillin-G (10µg), gentamicin (10µg), norfloxacin(10µg), oxytetracyclin (30µg), co-trimoxazole (25µ), and doxycycline hydrochloride (30µg) were place on plate. The plates were incubated for 18-24 h at 37°C. The zone of inhibition was measured and results were interpreted according to the guidelines of CLSI, (2014). The result obtained from the antibiotic sensitivity testing of all *E. coli* isolates were analyzed

for categorization of isolates as MDR *E. coli* or not MDR *E. coli* based on the recommendations of Magiorakos *et al.* (2012). They defined MDR as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. In this study, a total of six antimicrobial categories including nine antimicrobial agents were used to classify the isolates as MDR (Magiorakos *et al.*, 2012).

Results and Discussion

The focus of current study was to evaluate the association of *E. coli* with bovine clinical conditions and to generate its antibiotic sensitivity profile. In this study, a total of 200 bovine clinical samples including urine, milk and uterine discharge were subjected to isolation of *E. coli* by enrichment and selective plating. By enrichment in MacConkey broth with incubation at 44°C for 24 hr followed by streaking on the Eosin methylene blue agar (EMB agar) at 37°C for 24 hr, typical colonies of *E. coli* (purple with black centre and green metallic sheen colonies) were produced by 90 samples (45.00%). Upon confirmation by biochemical test, 71 isolates of presumptive *E. coli* on EMB agar were positive for characteristics biochemical reactions. These isolates were further screened for molecular confirmation of *E. coli* by detection of the presence of 16S *rRNA* gene by PCR (fig 1). This showed a distribution of *E. coli* in 35.50% (71/200) of total bovine clinical samples. The urine samples showed a distribution of *E. coli* in 45.67% (58/127) samples. In milk samples the distribution of *E. coli* was found as 19.05% (12/63) whereas, the distribution of *E. coli* in uterine discharge was found to be 10.00% (01/10).

The prevalence of *E. coli* in clinical bovine urine samples recorded in the study

(45.67%) was in concordance with the earlier reports of Sharma *et al.* (2006) who isolated *E. coli* in 57.10% cases of uroperitoneum. Kushwaha *et al.* (2012) analysed 31 urine samples from buffalo calves suffering from obstructive urolithiasis, and reported a prevalence of 16.12% *E. coli* which is much lower than the present findings. The prevalence of *E. coli* in clinical milk samples (19.05%), reported in the study, was in concordance with the earlier reports of Sumathi *et al.* (2008) and Lamey *et al.* (2013) as they isolated *E. coli*, from 20% and 18.47% samples, respectively. *E. coli* has been successfully isolated from bovine mastitis cases by number of investigators in India (Ranjan *et al.*, 2011; Kurjogi and Kaliwal, 2011; Hegade *et al.*, 2012; Palaha *et al.*, 2012) and abroad (Alekish *et al.*, 2013; Tesfaye *et al.*, 2013; Mahamoud *et al.*, 2015; Iraguha *et al.*, 2015). These investigators have recorded prevalence of *E. coli* from bovine mastitis in the range of 6-35 %. The isolation rate of *E. coli* from bovine clinical uterine discharge samples (10%) was somewhat in accordance with Azawi, (2008), who reported 18.4% isolation rate of *E. coli* from buffalo uterus. Ingale *et al* (2016) and Udhayavel *et al.*, (2013) reported 24.27% and 36.66% prevalence of *E. coli* in uterine washing and clinical cases of endometritis, respectively. The increased incidence of *E. coli* in uterus might be due to unhygienic practices during artificial insemination and during parturition results in contamination of uterus with dung, which is the main source for *E. coli*.

The antibiotic sensitivity shown by isolates is presented in table 2. More than 50% of isolates from urine showed resistance to Ceftriaxone, Oxytetracyclin, Penicillin, Cefotaxime and Cefotaxime/ Clavulanic acid. Similarly, a very high percentage of isolates showed resistance to these antibiotics. Amikacin and Chloramphenicol

are two antibiotics to which more than 80% of isolates from urine, milk and uterine discharge have shown sensitivity. The high prevalence of penicillin, cefotaxime/ clavulanic acid and cefotaxime resistant strains as observed in this study suggests that bovine can be a significant reservoir of penicillin and cefotaxime or cefotaxime /clavulanic acid resistant *E. coli* (Table 2). Intermediate resistance refers to those *E. coli* species that were not clearly resistant or susceptible (Adzitey, 2015). A good number of clinical samples have shown sensitivity to various antibiotics in intermediate range. The organisms that exhibit intermediate resistance also have the higher tendency to easily become resistant. The antimicrobial resistance study of *E. coli* isolates of uterine discharge revealed a high resistant or sensitivity percentage due to the number of *E. coli* isolated and used under the study was only one. The result of the finding of present study was somewhat supported by the findings of others with respect to antibiotic sensitivity profile of *E. coli* isolates. Rangel and Marin, (2009) tested 231 *E. coli* isolates from bovine mastitic milk and reported a high antimicrobial drug resistant especially for amoxicillin + clavulanic acid (85.7%), ceftriaxone (82.2%) and cotrimoxazole (68.8%) that was somewhat similar to the finding of present study. Chandrasekaran *et al.* (2014) also studied the prevalence of drug resistant *E. coli* isolates of mastitis in Tamil Nadu and reported a resistance to amoxicillin (53%), oxytetracycline (58%), penicillin G (60.5%), oxacillin (56.3%), gentamicin (43.7%), enrofloxacin (43.7%), amoxicillin + sulbactam (49.6%) and ceftriaxone (13.4%). In other study, Ingale *et al.* (2016) performed antibiotic sensitivity study on *E. coli* strains isolated from uterus of buffaloes and determined its in-vitro sensitivity to commonly used antibiotics. They reported antibiotic sensitivity to

tetracycline (100%), cotrimoxazole (100%), gentamicin (90%) and chloramphenicol (88%) with quite resistant to nitrofurantoin (48%) and amoxicillin (41%).

Magiorakos *et al.* (2012) defined MDR as non-susceptibility to at least one agent in three or more antimicrobial categories. Based on the finding of present study, a total of 66.20% isolates of bovine clinical samples were categorized as MDR (resistant to ≥ 3 to 6 antimicrobial categories). The prevalence of MDR *E. coli* in bovine clinical samples was 68.93 %, 50.00% and 100% in urine, milk and uterine discharge samples, respectively. In this study, among 58 *E. coli* isolates from clinical urine samples 22.41%, 18.96%, 18.96% and 8.6% were resistant to 3, 4, 5 and 6 anti microbial categories, respectively. While, out of 12 *E. coli* isolates from clinical milk samples 33.33%, 8.33% and 8.33% were resistant to 3, 4 and 5

antimicrobial categories, respectively. A single isolate from uterine discharge showed a resistant with 5 antimicrobial categories. The prevalence of MDR *E. coli* in clinical milk samples (50.00%) was nearer with the earlier reports of Todorovic *et al.* (2017) who 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 a prevalence of 45.83% isolates as MDR. A slight lower prevalence of 40% MDR *E. coli* isolates of healthy lactating cattle was reported by Sawant *et al.*, (2007). Brennan *et al.* (2016) isolated 150 *E. coli* from faecal samples collected from cattle with suspected enteric infection or milk-aliquots collected from cattle with suspected mastitis in France and Germany and expressed MDR in 30% isolates as resistance to ≥ 3 drug classes.

Table.1 Primer sequence used

SL. No	Primer Sequences (5' - 3')	Target gene/locus	Expected Product size (bp)	References
1.	ECA75F: GGAAGAAGCTTGCTTCTTTGCTGAC	16srRNA	544	Sabat <i>et al.</i> , 2000
	ECR619R: AGCCCGGGGATTTACATCTGACTTA			

F: Forward primer, R: Reverse primer

Fig.1 Confirmation of *E. coli* by amplification of 16SrRNA

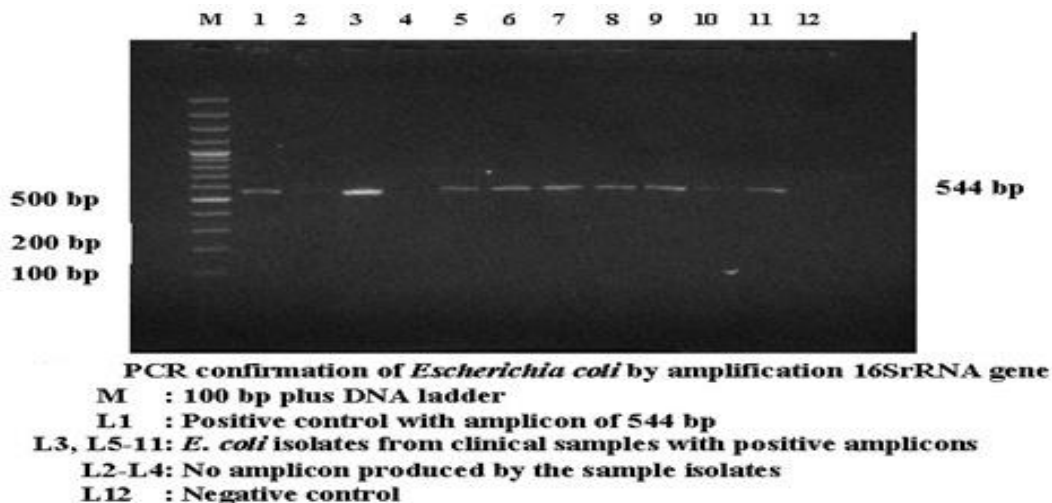


Table.2 Antimicrobial sensitivity pattern of *E. coli* isolates

Antibiotics	No of Isolates (%)								
	Urine			Milk			Uterine Discharge		
	R	IR	S	R	IR	S	R	IR	S
CIP	48.27	3.44	48.27	16.67		83.33	100	-	-
CTR	67.24	10.34	22.41	41.67	16.67	41.67	100	-	-
DO	24.13	32.76	43.10	16.67	16.67	66.67	100	-	-
GEN	20.69	20.69	58.62	16.67	25.00	58.33	100	-	-
COT	39.65	5.17	55.17	16.67	8.33	75.00	0	-	100
O	60.34	-	39.65	41.67	16.66	41.67	100	-	-
P	98.27	-	1.72	100	-	-	100	-	-
C	5.17	10.34	84.48	8.33	-	91.96	0	-	100
AMC	34.48	36.21	29.31	16.67	16.67	66.67	0	100	-
AK	6.89	5.17	87.93	8.33	-	91.66	0	-	100
CTX	81.03	12.07	6.89	66.67	16.67	16.67	100	-	-
NX	43.10	5.17	51.72	8.33	8.33	83.33	100	-	-
OF	41.38	3.44	55.17	8.33	-	91.66	0	-	100
A/S	6.89	43.10	50.00	-	41.67	58.33	100	-	-
CEC	82.76	-	17.24	83.33	-	16.67	100	-	-

*R= Resistant, IR= Intermediate Resistant, S= Sensitive

CIP=Ciprofloxacin, CTR=Ceftriaxone, DO=Doxycycline Hydrochloride, GEN= Gentamicin, COT= Co-Trimoxazole, O= Oxytetracycline, P= Penicillin G, C= Chloramphenicol, AMC= Amoxycillin/ Clavulanic acid, AK= Amikacin, CTX=Cefotaxime, NTX= Norfloxacin, OF= Ofloxacin, A/S= Ampicillin/ Salbactam, CEC= Cefotaxime/ Clavulanic acid

The higher prevalence rate of *E. coli* observed in the present study underscores the role of this pathogen in bovine urinary tract and udder infections. Although, the role of *E. coli* as a cause of environmental mastitis is well documented, the reports on its involvement in urinary tract infection in bovine are meagre. The most common cause of cystitis, urethritis and pyelonephritis in cattle is considered to be *Corynebacterium* species viz., *Corynebacterium renale*, *Corynebacterium cystidis* and *Corynebacterium pilosum* (Wallace *et al.*, 1990). In this study, the isolation of *E. coli* from 45.67% of urine sample indicates that the role of *E. coli* in setting urinary tract infections in cattle is highly under reported. Rebhun *et al.*, 1989, in a study to investigate the cases of pyelonephritis in cattle, isolated *E. coli* in 9 (9/15) and *Corynebacterium*

renale in 6 (6/15) urine samples. Similarly, Yeruham *et al.*, 2006, reported *E. coli* as most frequent cause of UTI in calves (29%) and in cows (31 %). The results of present finding are in agreement with these reports. Although, there is no dearth of reports that claims uropathogenic *E. coli* as primary pathogen involved in UTI cases in human (Hadifar *et al.*, 2017), it was surprising to find only three reports that indicated involvement of *E. coli* as a cause of UTI infections in cattle. This is even more intriguing considering the presence of *E. coli* in animal's intestine as a part of normal flora, its high build in the animal's surroundings, known ability of *E. coli* to colonise urinary tract and possession of virulence factors capable of inducing pathology in urinary tract. Thus, investigations in this direction to ascertain

role of *E. coli* in establishing UTI infections in cattle is highly warranted to appreciate the actual scenario.

Reports from world over showing rapid rise in emergence of antibiotic resistant *E. coli* strain from various sources viz., human UTI infections, sepsis and septic shock (Iredell *et al.*, 2016), samples from meat producing animals (Marshall and Levy, 2011; Onen *et al.*, 2015) and interestingly from environmental samples (Stephanie, 2015). The use of antibiotics in the treatment of diseases and as growth promoters in farm animals and other factors have been linked to the development of resistant microorganisms (Krumperman, 1983; Schroeder *et al.*, 2002; Aarestrup *et al.*, 2008; Adzitey *et al.*, 2012b). The *E. coli* isolates showing very high resistance to some of the antibiotics and high prevalence of MDR strains, as found in the present study, is pointing towards an alarming situation that warrants further investigations and instituting the frameworks to address the issue.

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