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Research Article

STATUS OF MULTIDRUG RESISTANCE AMONG ESBL PRODUCING *E. COLI* AND *KLEBSIELLA* SPP. ISOLATES OF BUFFALO ORIGIN IN EASTERN PLAIN ZONE OF UTTAR PRADESH

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ABSTRACT

Extended-spectrum β-lactamase producing organisms are expanding rapidly throughout the world and have become a problem for both veterinary and human medicine. Among family Enterobacteriacae, *Escherichia coli* and *Klebsiella* are of major public health concern. In the present study, a total of 240 buffalo milk and faecal samples were collected from two districts of eastern plain zone of Uttar Pradesh (India). Total 59.58% isolates were identified including 53.33% *E. coli* and 6.25% *Klebsiella* spp. by PCR, out of which 41.25% isolates were confirmed as ESBL producers comprising 36.66% *E. coli* and 4.58% *Klebsiella* species by phenotypic confirmatory tests. All ESBL positive isolates were found 100% sensitive to aminoglycosides and polypeptide class of antibiotics and 100% resistant to 3rd generation cephalosporins and ampicillin. Out of total ESBL producers, 78.0% isolates were found multidrug resistant. The present study revealed predominance of ESBL as well as MDR in *Klebsiella* spp. as compared to *E. coli* and highlights a great threat for horizontal gene transfer.

Keywords: Antibiogram, Buffalo, *E. coli*, ESBLs, *Klebsiella*

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Today, antimicrobial resistance (AMR) has been a global problem contributing to rise in cost of treatment and mortality (Singla *et al.*, 2014). The problem of AMR is expanding rapidly throughout the world and has become a problem for both veterinary and human medicine (Reist *et al.*, 2013). Extended-spectrum β-lactamases (ESBLs) are enzymes that can hydrolyse most of the β-lactam antibiotics and thus mediate resistance to penicillins, 3rd and 4th generation cephalosporins (Saravanan *et al.*, 2018). ESBLs are most commonly detected among Enterobacteriaceae like *E. coli* and *Klebsiella* spp. Extensive use of antibiotics leads to the development of multidrug resistance (MDR) organisms. MDRs in Enterobacteriaceae is increasing day by day that lead to limited antimicrobial treatment options which are posing a treatment challenge, and a major cause of morbidity and mortality worldwide (WHO, 2016).

Due to paucity of data on ESBL and MDR index in Eastern Plain Zone of Uttar Pradesh, the present study focussed on the MDR pattern among ESBL producing *E. coli* and *Klebsiella* spp. isolates from milk and faecal samples of healthy and diseased buffaloes of eastern plain zone of Uttar Pradesh (India). It will help the researchers, field veterinarians and farmers in selection of antibiotics for therapeutic purpose.

MATERIALS AND METHODS

Sample collection : Total 240 samples (120 milk samples and 120 faecal samples) were collected between August 2019 to June 2020 from 5 tehsils of Ayodhya and 3 tehsils of Sultanpur district of Eastern Plain Zone of Uttar Pradesh, India. Samples were collected randomly from apparently healthy and clinically infected animals and sampling consisted of 10 normal and 5 mastitic milk samples from each of the tehsil. Likewise, 10 normal and 5 diarrhoeic faecal samples from above mentioned regions were collected. Collected samples were immediately transported to Veterinary Microbiology Laboratory under cold condition for further processing.

Isolation and Identification : Samples were enriched with 2 ml nutrient broth and incubated for 24 hrs at 37 ºC. A loopful of inoculum was directly streaked on MacConkey agar (MLA) plates and incubated at 37 ºC for 24 hr. Colonies showing rose pink colour were picked up and transferred to nutrient agar slant and incubated at 37 ºC for 24 hrs. Thereafter, cultures were streaked on Eosine Methylene Blue (EMB) agar plates and colonies showing specific characteristics were identified by the method of Cruickshank *et al.* (1975). Further identification of the isolates was done using Gram's staining and standard biochemical tests, viz., IMViC pattern, catalase, nitrate reduction, urease, triple sugar iron agar and sugar fermentation as per method of Edward and Ewing (1972).

Extraction of genomic DNA : DNA templates were prepared by using snap-chill method as described by

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Franco *et al.* (2008).

Molecular identification of isolates : All presumptively positive *E. coli* isolates were confirmed by PCR amplification, using species specific uidA and *Klebsiella* by this bacterium specific 16S rRNA gene as per method described by Anbazhagan *et al.* (2010) and Andersson *et al.* (2008), respectively (Table 1). The cycling conditions of PCR are mentioned in Table 1.

Screening of isolates : All the confirmed *E. coli* and *Klebsiella* spp. isolates were subjected to ESBL screening, using 3rd, 4th generation cephalosporins and monobactam as per Kirby-Bauer's disk diffusion method (Fig. 5). The results were interpreted as per CLSI (2019) guidelines. The isolates showing resistance to any of these agents were further subjected to confirmatory phenotypic tests.

Confirmation of ESBL producing isolates by phenotypic methods :

Double disc synergy test (DDST): Isolates presumed as ESBL producer in screening, were further confirmed by DDST using ESBL kit 1 and Kit 3 (Hi-media) (Fig. 6). The commercially available discs were placed at 25 mm apart on Muller Hinton agar (MHA) plates inoculated with 1.5×10^8 organism/ml and incubated at 37 ºC for 24 hrs. The results were interpreted as per CLSI guidelines (2019).

Minimum inhibitory concentration (MIC) ESBL E-test : This test was done by placing strip on MHA plates inoculated with 1.5×10^8 organism/ml and incubated at 37 ºC for 24 hrs. The result was interpreted as per CLSI guidelines (2019) (Fig. 7).

Study of Multi-drug resistance (MDR) pattern: All the phenotypically confirmed ESBL isolates of *E. coli* and *Klebsiella* were checked for their resistance against 20 antibiotics of 12 different classes (Table 4). It was performed by disc diffusion method on MHA plates inoculated with 1.5×10^8 organism/ml and results were interpreted by the CLSI (2019) guidelines. Isolates showing resistance to at least one antibiotic in three or more classes of the drug were defined as MDR (CLSI, 2019).

RESULTS AND DISCUSSION

In this study, a total of 240 samples comprising 120 milk samples and 120 faecal samples were processed for isolation and identification (Fig. 1 & 2). On the basis of biochemical characteristics, 147 (61.25%) isolates were identified as *E. coli* and 19 (7.91%) isolates were presumed as *Klebsiella* spp. (Table 2). PCR analysis, confirmed total 128 (53.33%) *E. coli* and 15 (6.25%) *Klebsiella* spp., respectively (Table 2, Fig. 3 & 4). This finding was found in agreement with observations of various workers (Batabyal *et al.*, 2018; Ibrahim *et al.*, 2018). Higher isolation rate of *E. coli* in this study attributed to high prevalence of *E. coli* in the gut flora of ruminants.

Table 1

Oligonucleotide primer sequences used for amplification of *uid*A and *16S* rRNA genes and PCR cycling conditions used.

Targeted gene	Primer sequence (5'-3')	Amplicon size (bp)	PCR conditions and cycles	References
*uid*A *E. coli* specific	F- 5'CTGGTATCAGCGCGAAGTCT3'R R -5'AGCGGGTAGATATCACACTC3'	556	1 cycle of 5 minutes at 95 ºC, 35 cycles of 45 seconds at 95 ºC, 55 seconds at 56 ºC, 1 minutes at 72 ºC, 1 cycle of 7minutes at 72 ºC	Anbazhagan *et al.*, 2010
784F 1061R *Klebsiella* specific	F 5'AGGATTAGATACCCTGGTA3' R 5'CRRCACGAGCTGACGAC3'	265	1 cycle of 5 minutes at 95 ºC, 35 cycles of 50 seconds at 95 ºC, 45seconds at 54 ºC, 1minutes at 72 ºC, 1 cycle of 7 minutes at 72 ºC	Andersson *et al.*, 2008

Table 2

Isolation rate of *E. coli* and *Klebsiella* spp. in apparently healthy and clinical samples of Buffaloes origin

Samples (Source/Origin)	Presumptive positive isolates (Biochemical tests)			Confirmed positive isolates (PCR analysis)		
	E. coli	*Klebsiella* spp.	Total	*E. coli*	*Klebsiella* spp.	Total
Normal milk (n=80)	15 (18.75%)	05 (6.25%)	20 (25.0%)	11 (13.75%)	3 (3.75%)	14 (17.50%)
Mastitic milk (n=40)	21 (52.50%)	08 (20.00%)	29 (72.5%)	14 (35.00%)	7 (17.50%)	21 (52.50%)
Normal faecal (n=80)	73 (91.25%)	05 (6.25%)	78 (97.5%)	67 (83.75%)	2 (2.50%)	69 (86.25%)
Diarrhoeic faecal (n=40)	38 (95.00%)	03 (7.50%)	41 (102.0%)	36 (90.00%)	03 (7.50%)	39 (97.50%)
Total = 240	147 (61.25%)	19 (7.91%)	166 (69.2%)	128 (53.30%)	15 (6.25%)	143 (59.60%)

Table 3

Distribution of ESBL producing of *E. coli* and *Klebsiella* spp. among various sources

Samples (Source/Origin)	*E. coli* isolates	ESBL positive *E. coli*	*Klebsiella* spp. isolates	ESBL positive Klebsiella spp.	Total ESBL positive isolates
Normal milk (n=80)	11 (13.75%)	5 (6.25%)	3 (3.75%)	1 (1.25%)	6 (7.50%)
Mastitic milk (n=40)	14 (35.00%)	12 (30.00%)	7 (17.50%)	6 (12.50%)	18 (45.00%)
Normal faeces (n=80)	67 (83.75%)	51 (63.75%)	2 (2.50%)	2 (2.50%)	53 (66.25%)
Diarrhoeic faeces (n=40)	36 (90.00%)	20 (50.00%)	03 (7.50%)	2 (5.00%)	22 (55.00%)
Total = 240	128 (53.30%)	88 (36.66)	15 (6.25%)	11 (4.58%)	99 (41.25%)

Table 4

AMR pattern of ESBL positive *E. coli* and *Klebsiella* spp. isolates of buffalo origin

Group	Antibiotics (Hi-Media)	Conc. (µg/disc)	*E. coli* (n=88)	*Klebsiella* spp. (n=11)
			Resistance (%)	Resistance (%)
Aminogylcosides	Gentamicin (Gen)	10	0.0	18.18
	Amikacine (Ak)	30	0.0	0.0
Carbapenems	Imepenem (IMP)	10	10.23	36.36
	Meropenem (MRP)	10	4.55	18.18
3rd and 4th generation Cephalosporins	Cefotaxime (CTX)	10	100	100
	Cefpodoxime (CPD)	10	100	100
	Ceftazidime (CAZ)	30	76.14	72.73
	Ceftriazone (CTR)	30	100	100
Monobactams	Aztreonam (AT)	30	30.68	27.27
2nd generation Cephalosporins	Cefoxitin (CX)	30	14.77	27.27
Penicillin	Ampicillin (AMP)	25	100	100
Polypeptides	Polymyxin-B (PB)	300 unit	0.0	0.0
Sulphonamides	Co-trimoxazole (COT)	25	39.77	72.73
	Trimethoprim (TR)	30	27.27	27.27
Quinolones	Enrofloxacin (EX)	10	23.86	0.0
	Ofloxacin (OF)	2	20.45	9.09
	Nalidixic acid (NA)	30	46.59	45.45
Tetracycline	Tetracycline (TE)	30	22.73	27.27
Amoxyclav	Amoxicillin/Clavulanic (AMC)	(20/10)	4.55	45.45
Chloramphenicol	Chloramphenicol (C)	30	1.14	9.09

Present study also aimed to determine the proportion of ESBL resistant phenotypes among clinical and apparently healthy samples. Total 99 (41.25%) isolates were ESBL positive comprising 6 (7.50%), 18 (45.0%), 53 (66.25%) and 22 (55.0%) from normal milk, mastitic milk, normal faecal and diarrhoeic faecal samples, respectively (Table 3). These results were in accordance with the findings of Montso *et al.* (2019) and Yadav *et al.* (2019). ESBL positive *E. coli* (36.66%) were comparatively higher than ESBL positive *Klebsiella* spp (4.58%) which was not surprising since *E. coli* are dominant gut flora of ruminants. The occurrence of ESBL producers was found to be higher among mastitic milk isolates as compared to normal milk isolates irrespective of pathogen which may be attributed to indiscriminate and irrational use of antibiotics for treating mastitis in eastern plain zone of Uttar Pradesh. In this study too, the frequency of ESBL producer was higher in *Klebsiella* spp. isolates (11/15, 73.33%) than *E. coli* (88/128, 68.8%) which has been reported as major ESBL producer in other studies also (Prajapati *et al.*, 2020).

Antibiotic resistance is currently a serious problem that has received the attention of larger scientific community across the world. In present study, all the *E. coli* and *Klebsiella* spp. isolates were 100% resistant to 3rd, 4th generation cephalosporins and ampicillin. Both isolates of *E. coli* and *Klebsiella* spp. showed different susceptibility pattern with different classes of non-β

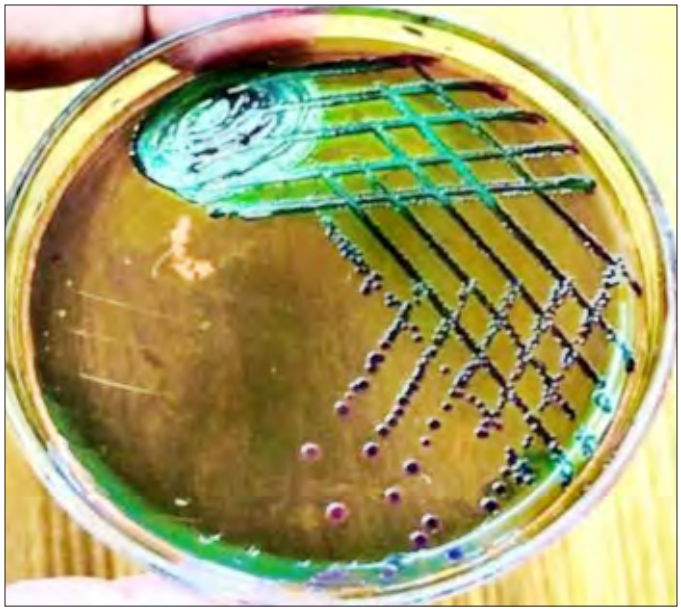

Fig. 1. Green metallic sheen of *E. coli* on EMB Agar

Fig. 2. Purple dark mucoid colony of *Klebsiella* spp. on EMB Agar

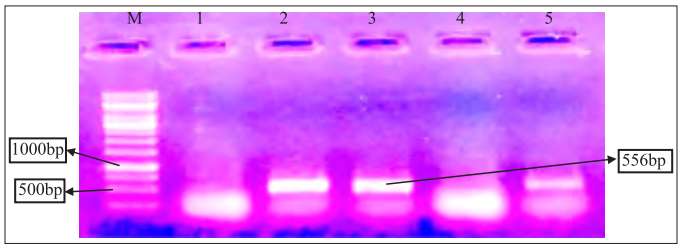

Fig. 3. PCR amplification of *uid* A gene for *E. coli* (556bp) M:1Kb ladder, Lane 2, 3 and 5 positive for *uid* A gene (556bp), Lane 1 and 4 negative for *uid* A gene

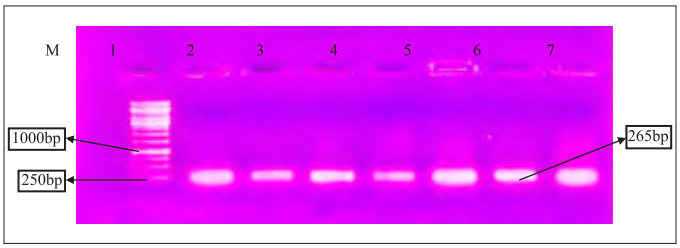

Fig. 4. PCR amplification of *16S* rRNA gene for *Klebsiella* (265bp) M:1Kb ladder, Lane 1, 2, 3, 4, 5, 6 and 7 positive for *16S* rRNA gene (265bp), Lane 1 and 4 negative for *uid* A gene

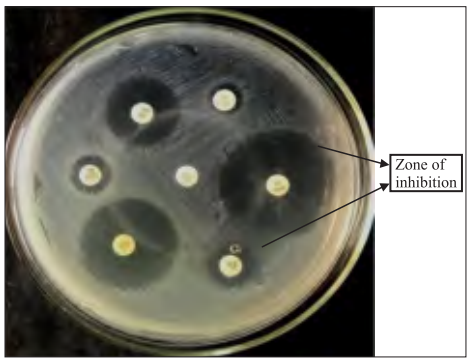

Fig. 5. Disc diffusion method for screening of ESBL producing *E. coli* and *Klebsiella* spp. isolates

Fig. 6. Double disc synergy test for confirmation of ESBL producing *E. coli* and *Klebsiella* spp.

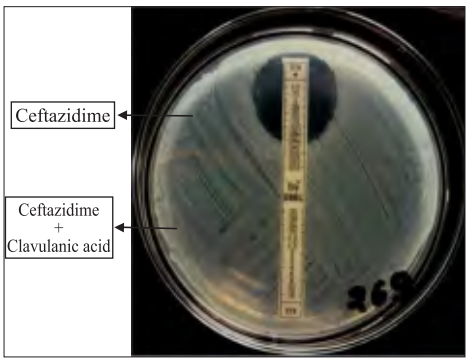

Fig. 7. ESBL E-strip test for confirmation of ESBL producing *E. coli* and *Klebsiella* spp.

lactam antibiotics except aminoglycosides and polypeptide class of antibiotics for which both isolates were found 100% sensitive (Table 4). There is abundant evidence to corroborate with the emergence of resistance against 3rd generation cephalosporins and ampicillin in India and abroad. Gupta *et al.* (2019) reported 63.7% resistance for ceftazidime, 94.1% and 93.1% sensitivity against gentamicin and colistin, respectively in *E. coli* isolated from neonatal faecal samples of calves. Batabyal *et al.* (2018) reported (60-100%) resistance against cefotaxime, ceftazidime, and 100% sensitivity to colistin, and imipenem in ESBL positive *E. coli* isolates of milk. Ibrahim *et al.* (2018) reported 100% resistance against ampicillin and 100% sensitivity against imipenem and meropenem in *E. coli* isolates of bovine milk. In this study, resistance to carbapenem antibiotics was also reported, even though these antibiotics are not being used in animal husbandry practices, this may be attributed to horizontal transfer of resistance gene between human and animal in community setting.

Multidrug resistant isolates is a cause of concern since they may pose severe health complications. In this study, MDR was assessed by calculating multiple antibiotic resistances (MAR) index of the isolates. Over all 36 resistance patterns were observed by 68 *E. coli* isolates ranging from 5 to 15 antibiotics and 8 resistance pattern by 9 *Klebsiella* spp. isolates ranging from 6 to 16 antibiotics. MAR index for *E. coli* and *Klebsiella* isolates was noticed in range of 0.25-0.75 and 0.30-0.80, respectively. This finding was in concordance with the finding of Mahato *et al.* (2019) and Sanjukta *et al.* (2019) with some variations. Total 78.0% ESBL positive isolates were found MDR which highlighted a potential threat by limiting the therapeutic options.

CONCLUSION

In this study, larger proportion of *E. coli* and *Klebsiella* spp. isolates showed ESBL as well as MDR phenotype. Most of the isolates showed resistance to 3rd - 4th generation cephalosporins and ampicillin, which is an alarming situation for this area. Despite this, some of the isolates of *E. coli* and *Klebsiella* also exhibited resistance against carbapenems even without its use in animal husbandry which is not a good sign from public health point of view.

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