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Prevalence, extended-spectrum β-lactamase and biofilm production ability of *Escherichia coli* isolated from buffalo mastitis

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ABSTRACT

This study aimed to determine the prevalence, antibiotic resistance pattern, extended-spectrum, beta-lactamase production and biofilm forming ability of isolated *E. coli* strains from buffaloes mastitis milk. Out of 549 bacterial isolates from mastitis milk of buffaloes (*n*, animal level= 472) between 2019 and 2022, a total of 43 *E. coli* strains were isolated with an overall prevalence of 9.11% at animal level. Prevalence of *E coli* was high in unorganised buffalo herd (11.36%) from villages of Farmer FIRST project (ICAR-FFP) compared with organised buffalo farms (6.73%). The highest resistance was against Penicillin 43 (100%) followed by Ceftriaxone 18 (41.86%), Amoxycillin/Sulbactam 8 (18.60%) and Enrofloxacin 7 (16.27%). Additionally, all were sensitive to gentamycin 43 (100%) followed by Cefoperazone/Sulbactam 34 (79.06%). Cephalosporins are frequently used antibiotics to treat bovine mastitis. However, their therapeutic effectiveness is being compromised by bacterial resistant to β-lactams. In present study, a total of 32 (6.78% %) extended-spectrum β-lactamase (ESBL) producing *E coli* were isolated from mastitic buffalo milk (n=43/472). In total, 17 (39.5%) isolates were biofilm producers by microtiter-plate method. There was statistically non-significant relationship between biofilm production and antibiotic resistance as well as between ESBL production and biofilm formation in *E coli* strains. Present study demonstrated a high occurrence of ESBL and biofilm producing *E. coli* in buffalo mastitis milk, implementing a significant challenge to treat mastitis in buffaloes, necessitates judicious use of antimicrobials and to explore potential therapeutic agents as substitutes for antibiotics to treat bovine mastitis effectively.

Keywords: Antimicrobial susceptibility test, Buffalo, Biofilm, *E. coli,* Farmer FIRST, Mastitis

Bovine mastitis is defined as inflammation of the glandular parenchyma of mammary gland that mainly occurs due to bacterial infection. The disease results in huge economic losses in terms of treatment cost, diagnostics, reduced milk yield and indirect losses such as culling and reduced conception rate of affected animals (Heikkilä *et al.* 2018). *E. coli* constitutes an extremely heterogeneous class of commensal gastrointestinal tract microbiota, however, pathogenic bacteria are competent of entering udder through fecal, water and bedding contamination of teat canal causing mastitis (Méric *et al.* 2016). Multidrug resistance (MDR) is defined as acquired non susceptibility to at least one antimicrobials agent in minimum three antimicrobial groups (Sweeney *et al.* 2018). *E. coli* producing ESBL are resistant to penicillin oxyimino cephalosporins and aztreonam, however, inhibited by beta-lactamase inhibitors such as clavulanic acid (Kayastha *et al.* 2020). Antimicrobial resistance (AMR) among *E. coli* has expended greatly in recent years, reducing the treatment options (Kayastha *et al.* 2020). Biofilm provides a potential virulence factors contributing to persistent infection, tissue damage and antimicrobial resistance (Di Domenico *et al.* 2017). Biofilm is a coherent community of microorganisms adhered in a biopolymer matrix, allowing enhanced resistance to antibiotics and evades host defense mechanisms compared to planktonic cells. *E. coli* intramammary infections are challenging to eliminate due to biofilm formation (Pedersen *et al.* 2021). Although, information about detection of ESBL producing *E. coli* are there among animals in India (Bandyopadhyay *et al.* 2018, Kuralayanapalya *et al.* 2019), a limited study has been reported on ESBL producing *E. coli* strains and their biofilm forming ability from milk of buffalo with mastitis (Yadav *et al.* 2022). Therefore, the present study was aimed to determine the prevalence of *E. coli* among other bacteria causing mastitis in buffaloes, antimicrobial susceptibility patterns, presence of ESLB production and phenotypic biofilm forming ability of *E. coli* isolated from buffaloes mastitis milk. Hence exploring biofilm and

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ESBL producing ability of *E. coli* in the study may provide alternate therapeutic strategies to prevent and treat mastitis.

MATERIALS AND METHODS

Bacterial strains and culture conditions: This study used 43 *E. coli* strains from a total of 549 bacterial isolates that were recovered from mastitis milk samples of buffaloes (n=472) between 2019 and 2022 at farmers doorstep from villages of Farmer FIRST project (ICAR-FFP) in Haryana and Rajasthan and organised herds of ICAR-CIRB. Bacterial isolation and identification was carried out on the basis of standard procedures Quinn *et al.* (1994).

Antimicrobial susceptibility test: Antimicrobial susceptibility was assessed on Mueller Hinton agar by disc diffusion method in accordance with the standards of Clinical Laboratory Standards Institute (CLSI 2012) using six different commercially manufactured antimicrobial discs (Himedia) from four antimicrobial classes constituting of gentamicin (GEN, 10 µg), enrofloxacin (EX,10 µg), ceftriaxone (CTR, 30 µg), cefaperazone/salbactum (CFS, 75/30 µg), penicillin-G (P,10 units/disc) and amoxicillin/ sulbactam (AMS, 30/15 µg). The antibacterial agents were chosen according to their presence in commercially available products for treatment of mastitis.

Extended-spectrum beta-lactamase (ESBL) detection: ESBL detection was performed in 2 steps, initially screening for potential ESBL producing strains was done for 43 *E. coli* strains using six antibiotic discs ceftazidime (CAZ, 30 µg), ceftriaxone (CTR, 30 µg), cefepime (CPM, 30 g), cefpirome (CFP, 30 µg), aztreonam (AT, 30 µg), cefpodoxime (CPD, 10 µg) on Muller-Hilton media plates inoculated with freshly growth bacterial culture (0.5 McFarland standard). The confirmative double-disk synergy test for ESBL was conducted if inhibition zone of these antibiotics on initial screening was ≤27 mm, ≤22 mm, ≤25 mm, ≤27 mm and ≤22 mm, respectively as per CLSI ESBL disc screening. The double-disk synergy test (Jarlier *et al.* 1988) was performed using CAZ, CTR, CPM, CFP, AT, CPD placed 20 mm apart from an amoxicillin (20 µg)/clavulanic acid (10 µg) disks on Mueller–Hinton agar plates. The plates are then incubated overnight at 37°C. Synergistic effect demonstrated by enhancement of one or more cephalosporin inhibition zone by ≥ 5 mm in presence of amoxicillin (20 µg) /clavulanic acid (10 µg) disks confirmed ESBL producing strains .

Phenotypic biofilm formation assay: Biofilm assay was carried out by quantitative microtiter-plate method (MPM), as reference standard for phenotypic biofilm screening in triplicate in 96-well sterile flat-bottomed microtiter plates with a lid (Nunc) as per (Stepanović *et al.* 2000). *E. coli* strains (n=43) were cultivated in 15 ml test tube with 10 mL of Tryptic Soy Broth (TSB) and incubated overnight at 37°C. The inoculum was adjusted to optical density $(OD)_{600}$ = 1. A total volume of 20 µL of inoculum and 180 µL of TSB was added to each well in triplicate, followed by incubation at 37°C for 24 h. After pouring off content from wells, biofilms were washed twice with distilled water to remove planktonic cells. Biofilm was fixed using 200 µL of 99% methanol per well for 15 min and allowed to air dry at room temperature for 60 min after emptying plate. Biofilms were stained with 200 µL crystal violet (1%) in each well for 20 min to quantify the total amount of biofilm biomass attached on the 96-well plate and then the excess of stain was rinsed off under tap water. Bound crystal violet adhered to cells was redissolved using 200 µL of 33% glacial acetic acid to each well. Absorbance was measured at 570 nm in an ELISA reader. A biofilm-forming strain (positive control) and 200 µL TSB (negative control) were used as controls during assay. The experiments were replicated thrice. The cut-off OD value (ODc): three standard deviations above the mean OD of the negative control, ODc = average OD of negative control + 3 (SD of negative control) where, SD = Standard deviation. Bacterial strains were classified into four classes namely non-biofilm producers, OD ≤ ODc; weak, OD > ODc and ≤ 2 × ODc; moderate, OD > 2 × ODc and ≤ 4 × ODc or strong biofilm producers with OD > 4× Odc

Statistical analysis: The chi-square test was used to find statistical relationship between categorical variables ($p<0.05$).

Table 1. Distribution of bacterial species and prevalence of mastitis from unorganised herd/ farmers' doorstep and organised herds at animal level

Pathogen	Unorganized buffalo herd, n=176	Organized buffalo herd, n=296	Total
	Numbers (%)*		
Staphylococcus aureus	10 (5.7)	17 (5.7)	27(5.7)
Coagulase-negative *staphylococci* (CoNS)	107 (60.8)	157 (53)	264(55.9)
Environmental *Streptococci*	10 (5.7)	30 (10.1)	40 (8.5)
Other gram positive	13 (7.4)	70 (23.6)	83 (17.6)
Pseudomonas spp	11(6.3)	30 (10.1)	41 (8.7)
Escherichia coli	20 (11.4)	23 (7.8)	43 (9.1)
Coliforms other than *Escherichia coli*	22 (12.5)	11 (3.7)	33 (7)
Lactose non-fermentor *Enterobacteriaceae*	14 (8)	4 (1.35)	18 (3.8)
Total isolates	207	342	549
Total samples	176	296	472

*Prevalence in percentage at animal level.

RESULTS AND DISCUSSION

Prevalence of E coli in organized and unorganized sector: Out of 549 bacterial isolates from subclinical and clinical mastitis milk samples of buffaloes from organised and unorganised herds analysed in the study (*n*, animal level = 472), a total of 43 *E. coli* strains were isolated. The overall prevalence of *E. coli* mastitis in buffalo was 9.1% at animal level, of which 11.4% and 7.8% were from unorganised and organised herds, respectively; no statistically significant difference was found ($\chi 2 = 1.54$, $p=0.21$) at $p \leq 0.05$ (Table 1). This is in close agreement with the study of Sharma *et al.* 2018 with record of 8.41% and in contrast with findings of Farooq *et al.* (2008), Bhanot *et al*. 2012, Singh *et al.* (2018) whose figures were 17.19%, 16.3% and 16%, respectively. However, prevalence of *coliform* mastitis was 16.1%, of which 23.9% were from unorganized and 11.5% from organised herds; a difference that was statistically significant ($\chi 2 = 11.58$, $p=0.0007$) at $p \leq 0.05$ (Table 1). A possible reason for high prevalence of *coliform* mastitis in unorganized herds may be poor milking practices and unhygienic environment around animal which increases predisposition to environmental mastitis. *E. coli* 43 (9.11%) was the third most prevalent pathogen after coagulase-negative staphylococci 264 (55.9%) and another gram positive 83 (17.6%).

Resistance profile of E. coli: The results of antimicrobial susceptibility testing of the 43 *E. coli* strains to 6 antibiotics are summarized in Table 2. The highest rate of *E. coli resistance* was found in P (97.67%), followed by CTR (41.86%), AMS (18.60%), EX (16.27%), respectively. A total of 2.3% isolates were only resistant to CFS. Multidrug resistant (MDR) pathogens are defined as acquired non susceptibility to at least one antibiotic agent in at least three antimicrobial groups. However, none of the *E. coli* isolate was found multidrug-resistant. The results revealed gentamicin as the most effective antibiotic against these strains with 97.67% susceptibility which is in agreement with the results of (Sumathi *et al.* 2008, Charaya *et al.* 2014, Bhat *et al.* 2017, Bisht *et al.* 2020). Indiscriminate continuous use, insufficient dose and incomplete course of treatment may be the reason for high antimicrobial resistance to commonly used ceftriaxone in buffalo mastitis. All the *E. coli* isolates were sensitive to gentamicin. On the contrary, previous reports showed most of *E. coli* strains, from bovine mastitis, resistant to gentamicin (Yang *et al.* 2018). This may indicate that short-term, limited use and low preference of gentamicin in mastitis treatment has contributed to the least resistance to this antimicrobial, among *E. coli* isolates.

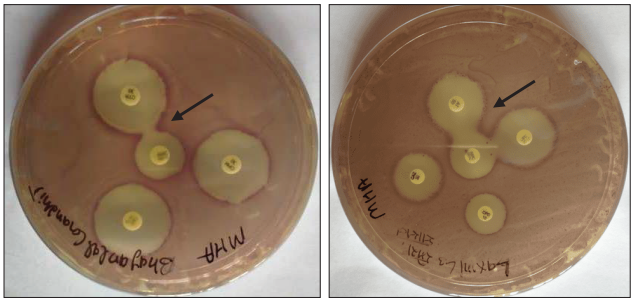

Fig.1. Double Disc Synergy Test (DDST) showing presence of Extended-spectrum ß-Lactamase (ESBL) positive clinical *E. coli* isolate using antibiotics CAZ, CTR, CPM, CFP, AT, CPD placed 20 mm apart from an amoxicillin/clavulanic acid disks on Mueller–Hinton agar plates. Black arrows show enhancement of the zone of inhibition in the DDST.

Extended-spectrum beta-lactamase (ESBL) detection: Out of 43 *E.coli* for ESBL screening, 41 isolates (95.4%) were resistant to at least one of the 6 screening antibiotics. Resistant isolates against CPM, CTR, CPD, CAZ, AT and CFP alone were 34, 35, 40, 33, 39 and 38, respectively. Twenty nine (67.44%) isolates were resistant to all the six antibiotics together. Out of 41 initial isolates screened positive for ESBL, an alarming rate of 32 (74.41%) *E. coli* were confirmed as ESBL producers by double disk synergistic ESBL phenotype confirmatory test using all 6 beta-lactam antibiotic-amoxicillin /clavulanic acid synergistic effect (Fig. 1, Table 4). Out of 472 clinical mastitis samples at animal level, 32 *E.coli* isolates with an overall prevalence of 6.78% were ESBL producers. These results were in accordance with the findings of (Kuralayanapalya *et al.* 2019) who reported a 9% overall prevalence of ESBLs in animal origin samples from India. Previous reports from various countries also showed a lower prevalence of ESBL-producing *Enterobacteriaceae* isolates (ranging from 0.4-13%) in cases related to bovine mastitis (Das *et al*. 2017). In India, scanty reports are available with reference to detection of ESBL producing *E. coli* in mastitis milk from buffalo with a substantial sample size (Yadav *et al.* 2022). The current study found an overall

Table 2. Antimicrobial susceptibility profile of *E. coli* isolates (n=43)

Class	Antimicrobials	Antibiotic sensitivity pattern, n=43		
		R	I	S
Penicillin with beta-lactamase inhibitor	AMS	8 (18.60)*	10 (23.25)	25 (58.13)
Penicillin	P	43 (100)	0 (0)	0 (0)
Aminoglycoside	GEN	0 (0)	0 (0)	43 (100)
Cephalosporin	CFS	1(2.3)	8 (18.60)	34 (79.06)
	CTR	18 (41.86)	5 (11.62)	30 (69.76)
Fluoroquinolone	EX	7 (16.27)	3 (6.97)	33 (76.74)

AMS (30/15 µg), amoxycillin/sulbactam; P(10 units/disc), Penicillin–G; GEN (10µg), gentamicin; CFS (75/30µg), cefoperazone/sulbactam; CTR (30µg), ceftriaxone; EX (10µg), Enrofloxacin.*Percentage in parentheses.

Table 3. Double disk synergistic test (DDST)* for phenotypic confirmation of ESBL producer *E.coli* (n=43) (Wayne 2010)

Double disk synergistic test	Positive isolates	Negative isolates
CPM/AMC	24	19
CTR/AMC	20	23
CPD/AMC	16	27
CAZ/AMC	26	17
AT/AMC	25	18
CFP/AMC	22	21
CPM/AMC, CTR/AMC, CPD/AMC, CAZ /AMC, AT/AMC and CFP/AMC together	32	11

AMC, (30/15 µg), Amoxicillin/ clavulanic acid.

prevalence of ESBL-producing *E. coli* to be about 6.78%. However, it highlights a significant high occurrence of ESBL-producing *E. coli* isolates 32 (74.41%), particularly in cases of *E. coli* mastitis, where the prevalence was 9.1% at animal level.

Phenotypic biofilm formation assay: In total, 8 (18.6%) isolates were strong biofilm producers, 5 (11.62%) moderate, 4 (23.5%) weak, with an overall 17 (39.5%) positive biofilm producers and 26 (60.4%) were biofilm non producers separated by microtiter-plate method (Fig. 2). Madani *et al.* (2022) reported 68.42% of 54 *E coli*

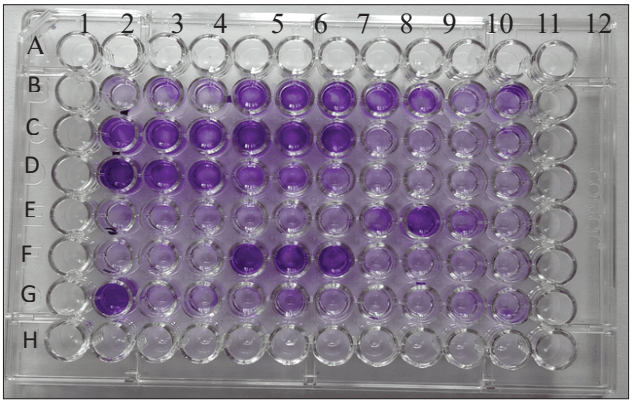

Fig. 2. Biofilm assay for *E. coli* isolates using 96-well microtiter plate method. B2, B3, B4: negative control (without inoculation); B5, B6, B7: moderate biofilm producers; D5, D6, D7: weak biofilm producers; D8, D9, D10: non-biofilm producer; F5, F6, F7: strong biofilm producers.

isolates from 200 milk and dairy product samples as biofilm producers. Resistance to cefepime (84.61 vs. 70.58%), ceftriaxone (88.46 vs. 70.58%), ceftazidime (76.92 vs. 76.47 %), cefpirome (88.46 vs. 88.23%), gentamicin (7.69% vs. 0%), enrofloxacin (23.07 vs. 23.52%), penicillin–G (96.15 vs. 94.11%), amoxycilin (42.3 vs. 35.29%) were comparatively higher among non-biofilm producing *E.coli* than biofilm producer, whereas, higher resistance to cefpodoxime (94.11 vs. 92.30) and aztreonam (94.11 vs. 88.46%) for biofilm producers compared to no biofilm produces (Table 4). Based on chi-square test, the relationship between biofilm production and antibiotic

Table 4. Antibiotic resistance pattern of biofilm forming and non-forming *E. coli* isolates (n=43)

Antimicrobial	Biofilm producer N=17	Biofilm Non-producer N=26	p-value
CPM	12 (70.58)*	22 (84.61)	0.27
CTR	12 (70.58)	23 (88.46)	0.14
CPD	16 (94.11)	24 (92.30)	0.82
CAZ	13 (76.47)	20 (76.92)	0.97
AT	16 (94.11)	23 (88.46)	0.53
CFP	15 (88.23)	23 (88.46)	0.98
G	0 (0)	2 (7.69)	-
CFS	3 (17.64)	7 (26.92)	0.48
EX	4 (23.52)	6 (23.07)	0.97
P	16 (94.11)	25 (96.15)	0.76
AMS	6 (35.29)	11 (42.30)	0.65

*Percentage in paranthesis.

resistance was found to be statistically non-significant for all antibiotics ($p>0.05$). Also, relationship between ESBL production and biofilm formation in *E coli* strains was statistically insignificant $X^2 = 0.10$, $p>0.05$ (Table 5). This was not similar to the observations (Neupane *et al.* 2016, Das *et al.* 2021) who reported significant association between biofilm production and antibiotic resistance, ESBL production and biofilm formation as well in uropathogenic *E. coli* strains from human.

The present study was the first attempt in comparing prevalence of *E. coli* mastitis in buffaloes from organised and unorganised herds, indicating a high prevalence of *coliform* mastitis from unorganised herds in ICAR-FFP villages. The finding emphasises the importance of field applicability of improving farmer's husbandry management practices particularly milking and environment of dairy animals in participatory mode, as a means to reduce exposure to environmental mastitis pathogens. is the present research is significant as it highlights the potential for antibiotic resistance and the ability of the studied bacteria to form biofilms, which contributes significantly to treatment failures. Gentamicin, cefoperazone/Sulbactam and enrofloxacin are the best choice of antibiotics, while amoxycillin/sulbactam and ceftriaxone antibiotics should be avoided against *E. coli* mastitis treatment in buffaloes from this region due to antibiotic resistance. Further studies are needed to study the efficacy of drug combination therapy including cephalosporins and amoxycillin/sulbactam for therapeutic management of mastitis and to develop more effective treatment. The molecular characterisation of these isolates at molecular level for presence of drug resistance

Table 5. Relationship between ESBL production and the ability to form biofilm among *E. coli* isolates (n=40)

ESBL Status	Biofilm producer	Biofilm non-producer	Total	p-value
ESBL producer	14	18	32	0.749
ESBL non-producer	3	5	8	
Total	17	23	40*	

*3 isolates sensitive in ESBL screening were excluded.

genes, ESBL and biofilm related genes can provide useful insights into the mechanisms of antibiotic resistance and biofilm formation in these bacteria.

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