Journal of Applied Life Sciences and Environment
https://jurnalalse.com

USV 1842

Article
https://doi.org/10.46909/alse-571122
Vol. 57, Issue 1 (197) / 2024: 19-36

RAW BOVINE MILK AS A RESERVOIR OF MULTI-DRUG RESISTANT, BETA-LACTAMASE-PRODUCING *Klebsiella*

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Received: Dec. 27, 2023. Revised: Jan. 28, 2024. Accepted: Jan. 30, 2024. Published online: Feb. 28, 2024

ABSTRACT. The transmission of zoonotic bacteria through consumption of raw milk is complicated by the dissemination of antimicrobial-resistant bacteria. The present study was conducted to detect the occurrence of antimicrobial-resistant bacteria (ESBL-/AmpC-producing *Klebsiella* spp.) in cow's milk originating from healthy or infected (mastitis) cattle in India. In total, 450 milk samples were collected from apparently healthy cattle and cattle suffering from clinical or sub-clinical mastitis. Out of 455 *Klebsiella* spp., 67 (14.73%) isolates were found to be ESBL producers in the double-disc diffusion test. The occurrence of ESBL-producing *Klebsiella* spp. was significantly ($p < 0.05$) higher in milk samples collected from cattle suffering with mastitis than in healthy cattle. Among the ESBL-producing *Klebsiella* spp., 56 (83.6%) isolates were also detected that produced AmpC β-lactamases. All the ESBL and AmpC-producing *Klebsiella* spp. possessed $bla_{CTX\text{-}M}$ (100%) and bla_{AmpC} (100%), respectively. The present study revealed a higher occurrence of class 1 integron in ESBL-producing *Klebsiella* spp. isolates. All ESBL-producing- *Klebsiella* spp. isolates were multi-drug resistant. The ciprofloxacin- and/or levofloxacin-resistant *Klebsiella* spp. isolates possessed the quinolone resistance gene (*qnrS*). The co-trimoxazole-resistant

Cite: Mahanti, A.; Joardar, S.N.; Bandyopadhyay, S.; Banerjee, J.; Ghosh, S.; Dutta, T.K.; Samanta, I. Raw Bovine Milk as a Reservoir of Multi-Drug Resistant, Beta-Lactamase-Producing *Klebsiella*. *Journal of Applied Life Sciences and Environment* **2024**, 57 (1), 19-36. https://doi.org/10.46909/alse-571122

isolates possessed the *sul1* and *sul2* genes. Phylogenetic analysis of the studied isolates revealed that strains isolated from the same location had a clonal relationship. The study increases consumer awareness of the need to avoid raw milk consumption to prevent the spread of antimicrobial resistance in the community.

Keywords: antimicrobial resistance; ESBL; *Klebsiella*; MIC; raw milk.

INTRODUCTION

Raw bovine milk as a reservoir of multi-drug resistant, beta-lactamase-producing *Klebsiella*

Food items such as milk function as ideal media for bacterial growth due to the presence of nutrients in optimum proportions. The microbes in raw milk originate from the bovine udder during intra-mammary infection (IMI) or diverse external sources such as air, contaminated hands of milkers, milking equipment, bulk tanks etc. The poor animal husbandry conditions associated with contaminated feed and drinking water, as well as the hind quarter hygiene of milch animals, play a significant role in the development of IMI, as environmental pathogens can enter the udder through the teat canal (Parekh and Subhash, 2008). The transmission of zoonotic bacteria through the consumption of raw milk and/or milk products is complicated by the dissemination of mobile genetic elements carrying antimicrobial resistance genes. The therapeutic efficacy and short withdrawal period of third- and fourth-generation cephalosporin β-lactam antibiotics make antimicrobial resistance a critical factor in dairy farming, especially during the treatment of mastitis, the most significant multi-etiological intramammary infection compromising the microbiological quality and quantity of produced milk. Overuse or misuse of antibiotics produces selection pressure that can generate commensal *Enterobacteriaceae*, including *Klebsiella pneumoniae*, that produce extended-spectrum β-lactamase (ESBL) or aminopenicillin-inactivating cephalosporinase (AmpC) (Liebana *et al.*, 2013). Data on the occurrence of ESBL-/AmpC-producing *Enterobacteriaceae*, particularly *Klebsiella pneumoniae*, in raw cattle milk are inconsistent (Gundogan and Yakar, 2007). Most of the studies published thus far have focused on milk samples collected from cattle suffering with mastitis, so their results cannot be extrapolated to apparently normal milk entering the food chain, collected from healthy cattle reared in the same geographical location (Dahmen *et al.*, 2013; Ohnishi *et al.*, 2013).

In the *Enterobacteriaceae* group of bacteria, including *Klebsiella* spp., *Escherichia coli* and *Salmonella* spp., extended-spectrum beta-lactamases (ESBLs) are major antimicrobial resistance determinants which are transmitted through horizontal gene transfer. The ESBL enzyme can produce resistance to a variety of β-lactam antibiotics, such as penicillins, higher-generation cephalosporins and aztreonam. Classical ESBL varieties include TEM (except TEM-1), SHV (except SHV-1 and 2) and CTX-M (EFSA, 2011). AmpC *β*-lactamase-producing organisms (ACBL) can generate resistance against *β*-lactamase inhibitors, such as clavulanic acid, in

addition to cephalosporins, penicillins, cephamycins and monobactams. Overexpression of the chromosomal AmpC gene (*bla*$_{AmpC}$) or possession of plasmid-mediated (CITM or *bla*$_{CMY-2}$) genes are found associated with the generation of resistance by ACBL producers (Schmid *et al.*, 2013). The emergence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE) is a cause of serious clinical and public health concerns, as carbapenem is considered as a last-resort therapeutic option against ESBL-producing bacteria. Carbapenem resistance in *Klebsiella* spp. is associated with the production of enzymes such as metallo-β-lactamase and *K. pneumoniae* carbapenemase (KPC) (Birgy *et al.*, 2012). Moreover, possession of ESBL determinants in bacterial plasmids is often associated with resistance to unrelated classes of antibiotics, for example, fluoroquinolones, aminoglycosides and sulfmethoxazole-trimethoprim (Coque *et al.*, 2008). Mobile genetic elements, such as class 1 integrons, aid in the transmission of ESBL-/ACBL-producing organisms (EFSA, 2011).

The Indian dairy sector is mostly unorganized, and farmers are the major stakeholders. The ownership of milch animals (cattle and water buffalo) is fragmented, and large numbers of farmers rear only a few animals (0.6–2.0 animals per holding) for milking and draught purposes (Landes *et al*., 2017). About 35% of the milk produced is collected by local co-operatives for processing and distribution to consumers, whereas the majority of the milk (40%) produced is consumed within the farmer's household or distributed locally without processing. During milk processing in dairy plants, the majority of bacteria, including *Enterobacteriaceae*, are destroyed at pasteurization temperature. The consumption of raw milk is still a traditional practice in Indian villages, and this practice has increased recently among urban youth, who believe in the beneficial health effects of raw milk. As the local food animals were found to harbour ESBL-/ACBL-producing *Enterobacteriaceae* (Bandyopadhyay *et al*., 2015; Kar *et al*., 2015; Samanta *et al*., 2015), animal products such as milk may constitute an important reservoir of ESBL-/AmpC-producing *Enterobacteriaceae*. The present study was conducted to detect the occurrence of antimicrobial-resistant bacteria (ESBL-/AmpC-producing *Klebsiella* spp.) in cow's milk originating from healthy or infected (mastitis) cattle in all agro-climatic zones of West Bengal, one of the major milk-producing states in India (Landes *et al*., 2017). The study also intended to reveal the occurrence of integron, production of carbapenemase and metallo-beta-lactamase enzymes, MIC of antimicrobials and phylogenetic relationships among the *Klebsiella* spp. isolates.

MATERIALS AND METHODS

Sampling

In the study, 450 milk samples were collected from apparently healthy (n = 168, without any visible clinical signs) cattle and infected cattle with clinical (n = 107) or sub-clinical mastitis (n = 175) from six agro-climatic zones (North 24 Parganas, Darjeeling, Coochbehar, Maldah, Mursidabad,

Bardhaman, Paschim Medinipur, Purulia, Purba Medinipur, South 24 Parganas districts) of West Bengal (India), irrespective of the age and breed of the animals, during 2016 (*Table 1*). The institutional animal ethics committee approved the study (WBUAFS/IAEC/032/2013-14, dated 22/10/2013). After discarding of the first two strips, the milk samples were collected directly into sterile vials. Sub-clinical mastitis was detected by using the California mastitis test (MAST INDEX, Tulip Diagnostics Private Limited, India) following the manufacturer's instructions. From the clinical mastitis cases, the milk samples were collected following the guidelines of the National Mastitis Council (NMC, 1990). Cattle without visible clinical signs and with a negative CMT were considered as 'apparently healthy'. A pooled milk sample was prepared by mixing all four quarter milk samples, and the pooled sample was brought into the laboratory, maintaining the cold chain.

Most of the cattle were reared in backyard farming systems where the farmers kept 2–3 cattle per household. All the cattle were milked by hand by the farmers. In cattle suffering with clinical mastitis, mostly higher generation cephalosporins (ceftizoxime) and sometimes enrofloxacin and tetracycline were used for therapy during milk sample collection. The data were collected from farmers during milk sample collection using the 'drug bag' method, where the bags containing the foils/vials of common antibiotics were displayed to the farmers to identify the antibiotics used in his/her cattle. Cattle suffering with sub-clinical mastitis were not treated, as no clinical signs were present.

Isolation, Identification and PCR-Based Confirmation of *Klebsiella* sp.

Klebsiella-selective agar (HiMedia, India) was used for isolation of the bacteria from the collected milk samples. More than one characteristic purple-magenta-coloured colony was selected and streaked on a nutrient agar slant (HiMedia, India) for morphological and biochemical confirmation. Morphological confirmation was performed by Gram staining and biochemical confirmation by indole, methyl-red, Voges-Proskauer and citrate utilization tests (Quinn *et al.*, 1994). PCR was used to confirm the isolates as *Klebsiella* spp. following the cycling conditions reported earlier, with some modifications (Brisse and Verhoef, 2001). The annealing temperature was modified using the positive control (ATCC-BAA-1705) in a gradient thermal cycler (Mastercycler Nexus, Eppendorf).

Double-Disc Diffusion Test

The disc diffusion assay with cefotaxime (HiMedia, India; 30 µg,) and ceftazidime (HiMedia, India; 30 µg,) with or without clavulanate (HiMedia, India; 10 µg,) was conducted to detect ESBL production by *Klebsiella* spp. isolates (Patel *et al.*, 2014). Phenotypic detection of AmpC production was conducted by cefoxitin-cloxacillin double-disc synergy (CC-DDS) (Tan *et al.*, 2009). PCR was used to confirm *Klebsiella pneumoniae* among all the ESBL-producing *Klebsiella* spp. isolates (Liu *et al*., 2008).

Table 1 – Isolation of ESBL-producing *Klebsiella* from cattle milk samples from different agro-climatic zones of West Bengal, India

Agroclimatic zone	Name of the District	No of Sample collected (No of samples positive for *Klebsiella* sp.)				No of *Klebsiella* sp. isolate identified (No of bla_{CTX-M} type ESBL Producing isolates)			
		CM	SCM	H	Total	CM	SCM	H	Total
Hill Zone	Darjeeling	15(12)	26(18)	19(10)	60(40)	24(0)	34(1)	18(3)	76(4)
Tarai Zone	Darjeeling	0(0)	10(6)	20(13)	30(19)	0	11(1)	23(6)	34(7)
	Coochbehar	0(0)	16(13)	20(12)	36(25)	0	25(1)	24(0)	49(1)
Old Alluvial Zone	Maldah	7(5)	8(7)	10(3)	25(15)	9(0)	14(4)	6(2)	29(6)
	Burdwan	10(6)	8(4)	5(1)	23(11)	12(10)	8(6)	2(0)	22(16)
New Alluvial Zone	Murshidabad	12(2)	18(4)	11(3)	41(9)	4(0)	8(4)	5(0)	17(4)
	Hooghly	7(0)	7(0)	27(2)	41(2)	0	0	4(0)	4(0)
Red and Laterite zone	Paschim Medinipur	10(9)	12(11)	14(5)	36(25)	18(1)	22(3)	10(0)	50(4)
	Purulia	23(8)	5(4)	10(3)	38(15)	16(3)	8(0)	5(0)	29(3)
	Purba Medinipur	0(0)	8(5)	12(4)	20(9)	0	10(2)	8(1)	18(3)
Coastal and Saline zones	North 24 Parganas	15(12)	28(20)	10(3)	53(35)	21(6)	37(4)	5(0)	63(10)
	South 24 Parganas	8(6)	29(21)	10(6)	47(33)	12(2)	41(6)	11(1)	64(9)
Total		**107(60)**	**175(113)**	**168(64)**	**450(238)**	**116(22)***	**218(32)***	**121(13)***	**455(67)**

CM: Clinical Mastitis, SCM: Sub-clinical Mastitis, H: Healthy
* Occurrence of ESBL-*Klebsiella* differs significantly ($p<0.05$)

Detection of Beta-Lactamase *(*Bla_{ctx-M}*,* Bla_{tem}*,* Bla_{shv}*)*, Chromosomal Bla_{ampc}, Plasmid-Mediated Ampc B-Lactamase (CITM) and Integron Genes in the *Klebsiella* spp. Isolates

PCR was conducted for detection of bla_{CTX-M}, bla_{TEM} and bla_{SHV} in *Klebsiella* spp. isolates showing a positive reaction in the double-disc diffusion test (Cao *et al.*, 2002; Weill *et al.*, 2004) Standard PCR for detection of bla_{AmpC} and CITM genes was also conducted in *Klebsiella* spp. isolates showing phenotypical AmpC production

(Féria *et al.*, 2002; van *et al.*, 2008). Class I integron was detected by PCR in all the ESBL-producing *Klebsiella* spp. isolates (Mazel *et al.*, 2000).

Antimicrobial Sensitivity of ESBL-Producing *Klebsiella* spp. Isolates

All the ESBL-producing *Klebsiella* spp. isolates were tested against different antimicrobials by the disc diffusion method (Patel *et al.*, 2014). The antimicrobial agents used were cefoxitin (30 µg), co-trimoxazole (25 µg), streptomycin (10 µg), ertapenem (10 µg), ceftriaxone/tazobactam (30/10 µg), ciprofloxacin (5 µg), tetracycline (30 µg), cefoperazone (75 µg), chloramphenicol (30 µg), cefepime (30 µg), gentamicin (10 µg), amikacin (30 µg), levofloxacin (5 µg), penicillin-G (10U), ampicillin/sulbactam (10/10 µg), doxycycline hydrochloride (10 µg), tobramycin (10 µg), ceftizoxime (30 µg), amoxycillin/clavulanic acid (20/10 µg), piperacillin (100 µg) and ceftazidime (30 µg) (HiMedia, India). The criteria for susceptibility/resistance were detected following the CLSI guideline (Patel *et al.*, 2014).

Phenotypic Detection of Carbapenemase and Metallo-Beta-Lactamase in ESBL-Producing *Klebsiella* sp. Isolates

All the ESBL-producing *Klebsiella* spp. isolates showing phenotypical resistance against ertapenem were subjected to the modified hodge test (MHT) and combination disc diffusion test (CDDT) (EDTA-750 µg + IMP-10 µg and IMP-10 µg) to confirm carbapenemase and metallo-beta-lactamase (MBL) production, respectively(Birgy *et al.*, 2012).

Detection of PMQR *(qnrA, qnrB, qnrS)* and Sulphonamide Resistance Genes *(sul1, sul2, sul3)*

The quinolone (*qnrA, qnrB, qnrS*) and sulphonamide resistance genes (*sul1, sul2, sul3*) were detected by PCR in all the ciprofloxacin- and/or levofloxacin- and co-trimoxazole-resistant ESBL-producing *Klebsiella* spp. isolates (Kar *et al*., 2015; Frank *et al.*, 2007).

Detection of Minimum Inhibitory Concentration of Cefotaxime, Ceftazidime, Ceftriaxone and Ampicillin

The MIC of cefotaxime, ceftazidime, ceftriaxone and ampicillin was determined against ESBL-producing *Klebsiella* spp. isolates using HiComb™ MIC Strip (HiMedia, India) and Ezy MIC strips (HiMedia, India) as per the guidelines of the manufacturer.

The MIC of ciprofloxacin was determined against *Klebsiella* spp. isolates possessing *qnr* genes using Ezy MIC paper strips (HiMedia, India) as per the guidelines of manufacturer.

Phylogeny of ESBL-Producing *Klebsiella* spp. Isolates

All ESBL-producing *Klebsiella* spp. isolates were typed by RAPD-PCR (Lim *et al.*, 2009), and images were analysed by using Doc-itLs image analysis software (UVP, UK). The phylogeny of the isolates was established by comparing the differences in the RAPD banding pattern. The neighbour joining method was used to construct an unrooted phylogenetic tree.

Statistical Analysis

The occurrence of ESBL-producing *Klebsiella* spp. in milk samples collected

from apparently healthy and infected cattle was compared by chi-square test and a descriptive analysis was performed using SPSS software (SPSS Inc.).

RESULTS AND DISCUSSION

The present study detected the occurrence of *Klebsiella* spp. in 238 (238/450, 52.8%) milk samples collected from healthy and diseased cattle. From the 238 samples, a total of 455 *Klebsiella* spp. isolates were identified phenotypically and confirmed by specific PCR. The isolation rate of *Klebsiella* spp. (38.09%–64.57%) varies according to the health status of the studied animals. Significant ($p < 0.05$) differences in the occurrence of *Klebsiella* spp. were detected between the three groups of cattle according to their health status (*Table 1*). More than one colony was selected from each composite milk sample; therefore, the isolated strains outnumbered the collected samples. The isolation rate (20%–60%) of *Klebsiella* spp. varied widely in raw milk samples collected from farmers, bulk tank milk and milk products (cheese, *khoa*) in Jordan, Sudan, Sri Lanka, Egypt and Turkey (Ahmed *et al.*, 2016; Badri *et al.*, 2017; El-Sukhon, 2003; Jayaweera *et al.*, 2018; Tepeli and Zorba, 2018). The existence of *Klebsiella* in the bovine udder is facilitated by the *lac* and *fec* iron-enterobactin operon, which helps *Klebsiella* spp. to utilize milk lactose more effectively than other commensal bacteria and might explain the high occurrence of the studied bacteria in the collected milk samples (Holt *et al.*, 2015).

Out of 455 *Klebsiella* sp. isolates, 67(67/455, 14.73%) were found to be phenotypical ESBL producers in the double-disc diffusion test. The frequency of ESBL-producing *Enterobacteriaceae* in raw milk from dairy farms varies from 0 to 9.5% throughout the world depending on husbandry conditions, hind quarter hygiene, the use of milking equipment and the use of antimicrobials on farms (Tepeli *et al.*, 2018; Geser *et al.*, 2012; Skočková *et al.*, 2015; Odenthal *et al.*, 2016). The studies with detection of ESBL-producing *Klebsiella* spp. in raw milk described a highly variable occurrence between 0 and 45% worldwide, which is concurrent with the findings of the present study (Gundogan and Yakar, 2007; Badri *et al.*, 2017; Özpınar *et al.*, 2017; Diab *et al.*, 2017). The occurrence of ESBL-producing *Klebsiella* spp. was significantly ($p < 0.05$) higher in milk samples collected from cattle suffering with mastitis (54/334, 16.1%) than in samples from healthy ones (13/121, 10.7%, *Table 1*). More samples were collected from infected cattle (clinical/sub-clinical mastitis) than from apparently healthy cattle, which may be one of the reasons, other than exposure to antibiotics, why more ESBL producers were obtained from infected milk samples. However, cattle with sub-clinical mastitis did not undergo any antibiotic therapy as they had no clinical signs. Earlier studies, including some form from India, also revealed mastitis milk as a potential source of ESBL-producing organisms due to high therapeutic antimicrobial exposure of the infected animals (Bandyopadhyay *et al.*, 2015; Locatelli *et al.*, 2010). Earlier studies detected

faecal carriage of ESBL/ACBL-producing *Enterobacteriaceae* by food animals in India (Kar *et al.*, 2015; Samanta *et al.*, 2015). Moreover, *Klebsiella* spp. isolated from human patients in West Bengal was detected to possess resistance determinants against higher-generation cephalosporins depicting the picture of the community carriage (Saha *et al.*, 2014), which indicates the milker's hands as another probable source of infection. Indeed, a limitation of the study is the lack of sampling from the milkers, which could successfully establish the hypothesis on the origin and transmission of *K. pneumoniae* clones.

Among the ESBL-producing *Klebsiella* spp., 56 isolates (56/67, 83.6%) were also confirmed as AmpC β-lactamase producers. The AmpC β-lactamase producing bacteria are although less common in nature but co-existence of ESBL and AmpC β-lactamase is a significant concern, as the co-existence of both resistance types exhibits a broader resistance profile (EFSA, 2011). AmpC β-lactamase-producing *Enterobacteriaceae* were detected earlier in raw milk and milk products (cheese) in different countries (Endimiani *et al.*, 2012; Özadam and Özpinar, 2016).

All the ESBL- and AmpC-producing *Klebsiella* spp. possessed $bla_{CTX\text{-}M}$ (67/67, 100%) and bla_{AmpC} (56/56, 100%), respectively, in PCR. Additionally bla_{SHV} (40/67, 59.7%) and bla_{TEM} (38/67, 56.7%) were detected in *Klebsiella* isolates. None of the isolates were positive for the plasmid-mediated AmpC β-Lactamase gene (CITM). In total, 17 (17/67, 25.3%) ESBL-producing *Klebsiella* isolates were confirmed as *Klebsiella pneumoniae* in PCR. Similarly, the predominance of CTX-M was detected earlier in *Enterobacteriaceae*, including *Klebsiella* spp. isolated from raw milk, bulk tank milk and dairy cattle throughout the world (Badri *et al.*, 2017; Dahmen *et al.*, 2013; Odenthal *et al.*, 2016; Schmid *et al.*, 2013). In previous studies from the same geographical location, CTX-M was predominantly detected among the ESBL-producing *E. coli* in cattle, poultry and pigs (Bandyopadhyay *et al.*, 2015; Kar *et al.*, 2015; Samanta *et al.*, 2015). Similarly, earlier studies in food animals and birds confirmed the distribution of bla_{AmpC} in this location (Banerjee *et al.*, 2019; Samanta *et al.*, 2018).

The present study revealed a higher occurrence of class 1 integron in ESBL-producing *Klebsiella* spp. isolates (53.7%), depicting their high transmission potential.

All ESBL-producing *Klebsiella* sp. isolates were resistant to ceftazidime, cefepime and penicillin-G but sensitive to chloramphenicol. Higher resistance was observed against piperacillin (97%), amoxycillin/clavulanic acid (94%), ceftizoxime (86.5%) and cefoperazone (85%), whereas a lower degree of resistance was found against amikacin (1.5%), tobramycin (3%), gentamicin (3%), ceftriaxone/tazobactam (7.5%), ampicillin/sulbactam (9%), ciprofloxacin (13.4%) and levofloxacin (13.4%). Similarly, the *Enterobacteriaceae* isolated from milk and milk products showed phenotypical resistance against penicillin G, cloxacillin, ceftriaxone, cefotaxime, ceftazidime, tetracycline, ciprofloxacin and norfloxacin (Badri *et al.*, 2017; Geser *et al.*, 2012; Osman *et*

al., 2014; Su *et al.*, 2016). The MIC of cefotaxime, ceftazidime, and ceftriaxone against ESBL-producing *Klebsiella* sp. isolates varied from 0.01 µg/ml to 240 µg/ml, 3 µg/ml to >240 µg/ml and 0.01 µg/ml to 240 µg/ml, respectively (*Table 2*). All the isolates were found to be multidrug resistant based on the criterion of resistance to at least one agent in three or more antimicrobial categories (Magiorakos *et al.*, 2012) (*Table 2*).

Table 2 – Antimicrobial resistance profile, *bla* genotyping and MIC values of different antibiotics against ESBL-producing *Klebsiella* isolated from milk samples in West Bengal, India

Isolate No.	Genotype	Antimicrobial Resistance Profile	MIC_{CTX} (µg/ml)	MIC_{CAZ} (µg/ml)	MIC_{CTR} (µg/ml)
K1	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC CZX PI CAZ	30	>30	>10
K2	*bla*$_{CTX-M}$	CPM P PI CAZ	30	30	5
K3	*bla*$_{CTX-M}$	CPM P CZX CAZ	10	30	>30
K4	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$	CPM P PI CAZ	30	30	5
K5	*bla*$_{CTX-M}$	CPM P AMC PI CAZ	30	>30	>15
K6	*bla*$_{CTX-M}$	ETP CPZ CPM P AMC CZX PI CAZ	30	30	30
K7	*bla*$_{CTX-M}$	ETP CPZ CPM P AMC CZX PI CAZ	30	30	30
K8	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT CPZ CX S CPM P AMC DO CZX PI CAZ	30	120	30
K9	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX CPM P AMC DO CZX PI CAZ	30	>30	30
K10	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX CPM P AMC DO CZX PI CAZ	30	>30	30
K11	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT CPZ CX S CPM P A/S AMC DO TOB CZX PI CAZ	30	>60	>30
K12	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX CPM P AMC DO CZX PI CAZ	30	>60	>30
K13	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT CPZ CX CPM P AMC DO CZX PI CAZ	30	>240	>15
K14	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX CPM P AMC DO CZX PI CAZ	30	120	30
K15	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT CPZ CX S CPM P AMC DO CZX PI CAZ	30	120	30
K16	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT CPZ CX S CPM P AMC DO CZX PI CAZ	30	>30	30
K17	*bla*$_{CTX-M}$ *bla*$_{TEM}$	COT CPZ CX CPM P AMC DO	30	>30	30

	bla$_{SHV}$ *bla*$_{AMPC}$ *intI sul1 sul2*	CZX PI CAZ			
K18	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX CPM P AMC DO CZX PI CAZ	60	30	30
K19	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT CPZ CX S CPM P AMC DO CZX PI CAZ	60	30	30
K20	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX CPM P AMC CZX PI CAZ	30	>240	>15
K21	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT CPZ CX S CPM P AMC CZX PI CAZ	30	>240	>15
K22	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC CZX PI CAZ	30	30	30
K23	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC CZX PI CAZ	30	30	30
K24	*bla*$_{CTX-M}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC CZX PI CAZ	30	30	30
K25	*bla*$_{CTX-M}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC CZX PI CAZ	30	30	30
K26	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC CZX PI CAZ	30	30	5
K27	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	ETP CPZ CX CPM P AMC CZX PI CAZ	10	30	>30
K28	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI*	ETP CPZ CX CPM P AMC CZX PI CAZ	10	30	>30
K29	*bla*$_{CTX-M}$ *bla*$_{SHV}$	ETP CPZ CPM P AMC CZX PI CAZ	30	30	30
K30	*bla*$_{CTX-M}$ *bla*$_{SHV}$	ETP CPZ CPM P AMC CZX PI CAZ	30	30	30
K31	*bla*$_{CTX-M}$ *bla*$_{SHV}$	ETP CPZ CPM P AMC CZX PI CAZ	>30	30	>240
K32	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$	CX CPM P AMC PI CAZ	0.1	7.5	0.01
K33	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1*	COT CPZ CX CPM P AMC CZX PI CAZ	120	240	30
K34	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1*	COT CPZ CX CPM P AMC DO CZX PI CAZ	120	240	30
K35	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul2*	COT ETP CIP CPZ CX CPM LE P AMC DO CZX PI CAZ	>30	>240	>240
K36	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul2*	COT ETP CIP CPZ CIT CX CPM TE LE P AMC DO CZX PI CAZ	>30	>240	>240
K37	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1*	COT CPZ CX CPM P AMC DO CZX PI CAZ	120	240	30
K38	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1*	COT ETP CPZ CIT CX CPM TE P AMC DO CZX PI CAZ	120	240	30
K39	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *sul1 sul2*	COT CPZ CX CPM TE P A/S AMC DO CZX PI CAZ	240	30	120

K40	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *sul1 sul2*	COT CPZ CX CPM TE P AMC DO TOB CZX PI CAZ	240	30	120
K41	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CIP CPZ CX CPM GEN TE LE P AMC DO CZX PI CAZ	30	>30	>10
K42	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CIP CPZ CX CPM GEN LE P AMC DO CZX PI CAZ	30	>30	>10
K43	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI qnrS sul1*	COT ETP CIP CPZ CX CPM LE P AMC DO CZX PI CAZ	120	240	30
K44	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI qnrS sul1*	COT ETP CIP CPZ CIT CX CPM LE P AMC DO CZX PI CAZ	120	240	30
K45	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX S CPM TE P A/S AMC CZX PI CAZ	60	30	30
K46	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX CPM TE P A/S AMC DO CZX PI CAZ	60	30	30
K47	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI qnrS sul1 sul2*	COT ETP CIP CPZ CX CPM TE LE P AMC DO CZX PI CAZ	>10	30	60
K48	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI qnrS sul1 sul2*	COT ETP CIP CPZ CX CPM TE LE P AMC DO CZX PI CAZ	>30	30	>30
K49	*bla*$_{CTX-M}$ *bla*$_{AMPC}$	ETP CPZ CX CPM TE P AMC DO CZX PI CAZ	120	120	60
K50	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI qnrS sul1 sul2*	COT ETP CIP CPZ CX CPM TE P AMC DO CZX PI CAZ	>30	30	>30
KP1	*bla*$_{CTX-M}$ *bla*$_{SHV}$	ETP CPM P AMC DO PI CAZ	10	30	>30
KP2	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CX CPM AK P AMC CZX PI CAZ	0.01	3	0.01
KP3	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	ETP CX CPM P AMC PI CAZ	30	30	5
KP4	*bla*$_{CTX-M}$ *bla*$_{SHV}$	ETP CPM P AMC PI CAZ	30	>30	15
KP5	*bla*$_{CTX-M}$ *bla*$_{AMPC}$	CX CPM P CAZ	0.01	3	0.01
KP6	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC DO CZX PI CAZ	30	>60	>30
KP7	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC DO CZX PI CAZ	30	>60	>30
KP8	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC DO CZX PI CAZ	30	30	30
KP9	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC DO CZX PI CAZ	30	30	30
KP10	*bla*$_{CTX-M}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC DO CZX PI CAZ	30	>30	30
KP11	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$	CPZ CX CPM P AMC DO CZX PI CAZ	30	>30	30
KP12	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI*	COT ETP CPZ CX S CPM P AMC CZX PI CAZ	30	120	30

	sul1 sul2				
KP13	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX S CPM P AMC DO CZX PI CAZ	30	>240	>15
KP14	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX CPM TE P AMC DO PI CAZ	0.01	3	0.01
KP15	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1*	COT ETP CPZ CIT CX CPM TE LE P AMC CZX PI CAZ	60	30	30
KP16	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CX CPM TE P A/S AMC DO CZX PI CAZ	60	60	30
KP17	*bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{SHV}$ *bla*$_{AMPC}$ *intI sul1 sul2*	COT ETP CPZ CIT CX S CPM TE P A/S AMC DO CZX PI CAZ	60	60	30

However, none of the ESBL-producing *Klebsiella* spp. isolates were positive for carbapenemase and metallo-beta-lactamase production in phenotypic assays. Overuse or misuse of antibiotics is associated with the generation of resistance against the antibiotics used in a particular setting. The multi-drug resistance profile of ESBL-producing *Klebsiella* spp. isolates showed the pattern of antibiotic exposure (cephalosporins, enrofloxacin and tetracycline, not carbapenem) of the studied cattle. Moreover, possession of *bla*$_{ESBL}$ genes in ESBL-producing *Klebsiella* sp. isolates indicated about the possession of single conjugative plasmid which may also carry the *sul* gene causing co-resistance against co-trimoxazole (Cantón and Coque, 2006).

Five out of the nine ciprofloxacin and/or levofloxacin resistant *Klebsiella* spp. isolates possessed plasmid-mediated quinolone resistance gene (*qnrS*). The MIC of ciprofloxacin against *qnr* positive *Klebsiella* spp. isolates was found to be >32 µg/ml (*Table 2*). Similarly, earlier studies showed an MIC of 33.3 µg/ml produced by *qnrS1* possessing *Enterobacteriaceae* isolates (van der Putten *et al.*, 2019). Among co-trimoxazole-resistant (n = 37) ESBL-producing *Klebsiella* sp. isolates, seven and two isolates were positive for the *sul1*and *sul2* genes, respectively, whereas, 28 isolates possessed both the *sul1*and *sul2*, but none of the isolates were positive for *sul3* in PCR.

All 67 ESBL-producing *Klebsiella* spp. isolates were typeable with the primers used in RAPD-PCR. The amplified fragment size ranged from 170 bp to 4178 bp (calculated by Doc-itLs image analysis software, UVP, UK). The phylogenetic analysis of the studied isolates revealed that the strains isolated from same district were grouped in same cluster, indicating their phylogenetic relationship (K6 and K7; K49 and K50; K8 and K14; K39 and K40; K37 and K44; KP7, KP8 and KP10; K9, K10, KP13 and K17; KP9 and KP11).

CONCLUSIONS

The present study revealed a moderately higher occurrence of multi-drug resistant ESBL-/AmpC-producing

Klebsiella spp. in raw milk collected from healthy as well as infected cattle. The occurrence of ESBL-producing *Klebsiella* spp. was significantly higher in milk samples collected from cattle suffering with mastitis than in those from healthy ones. Among the ESBL-producing *Klebsiella* spp., 56 isolates were also detected to produce AmpC β-lactamases. The present study detected the occurrence of class 1 integron in ESBL-producing *Klebsiella* spp. Isolates,87 depicting their high transmission possibility. The phylogenetic analysis of the studied isolates revealed that the strains isolated from same location had clonal relationship. The study made the consumers aware to avoid the raw milk consumption to prevent the spread of antimicrobial resistance

Author Contributions: AM, JB, SG collected the samples and conducted the methodology part. SNJ and IS supervised the study. IS conceptualized the study and wrote the primary manuscript. SB and TKD edited the manuscript. All authors declare that they have read and approved the publication of the manuscript in the present form.

Acknowledgement: The authors provide sincere thanks to Honourable Vice Chancellor, West Bengal University of Animal and Fishery Sciences for the infrastructure and facilities.

Funding: There was no external funding for this study.

Conflicts of Interest: The authors declare that there is no conflict of interest.

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Academic Editor: Prof. dr. Daniel Simeanu