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Antimicrobial resistance (AMR) pattern of milk borne *Staphylococcus* spp. and *Escherichia coli* in Jammu region

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ABSTRACT

Antimicrobial resistance is a public health hazard that affects humans, animals as well as the environment. Antibiotics are becoming gradually ineffective as drug-resistance spreads globally. The dairy industry is a major consumer of antibiotics globally and treatment of infected udder is the most common reason of use of antibiotics in cows. The study highlights the present status of microbial resistance to commonly used antibiotics in dairy animals of Jammu, a region in northern India. The study showed high prevalence of methicillin and tetracycline resistance among *E. coli* and *S. aureus* of udder origin. A high percentage (60%) of the isolates showed resistance to two or more than two classes of antibiotics with high prevalence of methicillin and tetracycline resistance. Methicilin resistance was more prevalent in Gram positive *Staphylococci,* whereas tetracycline resistance was frequent in both Gram positive *Staphylococci* and Gram negative *E. coli*. Abundance of tetracycline resistant (*Tet A, Tet O and Tet M*) and methicillin resistant (*MecA, MecI and MecR*) genes were fairly common in *E. coli* and *S. aureus* of udder origin.

Keywords: Antibiotic resistance, *E. coli,* Jammu region, *S. aureus,* Udder hygiene

A varied range of chemical substances are employed in antibiotics to either prevent bacterial growth (bacteriostatic) or to actually kill the bacteria (bactericidal). Bacteria, in turn, can employ a variety of mechanisms to render themselves less sensitive or insensitive to antibiotics leading to antimicrobial resistance (AMR). Microbes develop resistance against antibiotics either vertically through *de novo* mutations *e.g.* mutations in the gyrA and parC genes leading to a reduced binding affinity of fluoroquinolone (Willmott and Maxwell 1993) or horizontally by acquiring mobile antibiotic-resistance genetic elements such as plasmids and conjugative transposons (Von Wintersdorff *et al*. 2016). Another mechanism, commonly employed by multidrug-resistant (MDR) bacteria is acquiring *de novo* mutations that cause the overexpression of genes encoding efflux-pump proteins (Blair *et al*. 2015, Vidovic *et al*. 2019).

Antimicrobial resistance is listed as one of the top ten threats to global health (WHO 2019). AMR in humans is interlinked with AMR in other populations, especially farm animals, and in the wider environment (Woolhouse *et al*. 2015). Antimicrobials in livestock are predominantly used for therapy, prophylaxis, as growth promoters, and for preventing the spread of diseases in animals and poultry (Mcewen and Fedorka 2002, Boamah *et al.* 2016). However, indiscriminate and overuse without following the withdrawal period, contamination of animal feed with the excreta of treated animals, sub-standard quality of veterinary medicine, and the use of unlicensed antibiotics are a few of the factors that contribute to antimicrobial resistance (Chowdhury *et al*. 2015, Clifford *et al*. 2018). In September 2016, the United Nations (UN) General Assembly recognized the inappropriate use of antimicrobials in animals as a leading cause of rising AMR. The new WHO recommendations (WHO 2019) urge prudent usage of antibiotics to reduce the risk. Antibiotic resistance is a dynamic process and needs continuous monitoring to follow its proper pattern. Unlike human medicine, there is only limited public and private information on veterinary antimicrobial usage accessible to the public health community. The present study was undertaken to study the pattern of AMR in Gram-positive *Staphylococcus* spp. and Gram-negative *Escherichia coli* of milk origin in the Jammu district of India.

MATERIALS AND METHODS

Sample collection: A total of 100 milk samples were collected randomly from different households rearing dairy animals (herd size >10) in 13 villages and peri-urban areas of the Jammu region. Milk samples were preferably collected from quarters suspected of infection. In cases where all animals in the herd had apparently healthy quarters, the selection of quarters was random. Animals who were not undergoing an antibiotic course were chosen for sampling in the interim. The sampling steps included udder preparation, fore-stripping, aseptic collection of

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milk, and transportation of samples to the laboratory under refrigeration. Before collection, the udder was washed with clean water followed by drying with a clean towel. The first four strips of milk were discarded. Around 5 ml of milk was then collected in a sterile 15 ml conical tube. The milk samples were transported to the laboratory under refrigeration (4°C) and processed within 12 h of collection.

Screening and identification of Staphylococcus spp. *and Escherichia coli:* To screen *Staphylococcus* spp. and *Escherichia coli*, enrichment of milk samples was done. A loopful of milk samples was inoculated in the sterile enrichment medium (Tryptone soya broth; HiMedia Pvt. Ltd., India) and incubated for 24 h at 37°C. The culture was then streaked separately onto Baird Parker agar (HiMedia Pvt. Ltd., India) plates containing one of the antibiotics viz. tetracycline (20 µg/ml), oxacillin (1 µg/ml), ciprofloxacin (5 µg/ml), or ofloxacin (5 µg/ml). After 24 h of incubation at 37°C, colonies were Gram-stained and examined for morphology. Similarly, a loopful of inoculum from the enrichment broth was streaked on eosin methylene blue (EMB) agar (HiMedia Pvt. Ltd., India) containing the above-mentioned antibiotics. After 24 h of incubation at 37°C, colonies were Gram-stained and examined for morphology.

The identity of *Staphylococcus* spp. and *Escherichia coli* was confirmed through rDNA sequencing. For this, bacterial DNA was isolated by the standard phenol-chloroform method (Sambrook and Russell 2006). For partial amplification of rDNA, the universal primers were designed from available ESTs at the NCBI GenBank database (Table 1).

All the PCR amplifications were performed in 25 µl reaction volume. The reaction consisted of 100 ng template DNA, 2.5 µl of 10× buffer, 0.5 µl of 10 mM dNTP mix, 0.2 µl of Taq DNA (Kapa Biosystem, Merck KGaA, Darmstadt, Germany), 0.5 µl of each primer (10 pmol) and sterile nuclease-free water. The PCR conditions in the thermal cycler consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and a final extension step at 72°C for 5 min. The PCR products were resolved on agarose gel (2.5%). The PCR products were purified by Charge Switch PCR purification kit (Invitrogen, CA, USA). The purified products were sequenced from a commercial sequence service provider (Biologia Research India Pvt. Ltd.).

Screening of antimicrobial resistance: The antimicrobial resistance pattern of prevalent organisms was determined by the disk-diffusion method, determination of minimum inhibitory concentration, and amplification of resistant genes.

Disk diffusion assay: The disk diffusion assay of organisms was performed in Muller Hinton agar (MHA). Four discs were applied on a single plate. The antibiotic discs included those containing tetracycline, methicillin, ofloxacin, and ciprofloxacin. After overnight incubation at 37°C, inhibition zone diameters were interpreted and categorized as susceptible, intermediate, or resistant as per guidelines of the Clinical and Laboratory Standards Institute (CLSI 2020).

Minimum inhibitory concentration (MIC): The MIC of resistant organisms was determined by the broth microdilution method (Wiegand *et al*. 2008). All the MIC experiments were performed in a 96-well plate format. Each row of a 96-well plate contained two-fold serial dilution of one antibiotic (50 µl/well, up to 10^{th} well), one growth control, and one sterility check well. For each isolate, three to five isolated colonies of the same morphological appearance from the fresh agar plate were selected and transferred into a tube containing 3-4 ml of MHB. The lowest antibiotic concentration in the well that showed no visible turbidity was defined as MIC.

Partial Amplification of AMR genes: Primers for amplification of genes conferring resistance to methicilin, tetracycline, and quinolone group of antibiotics were designed from available ESTs and nucleotide sequences at NCBI (Table 2).

These primers were tested with *in silico* PCR and BLAST match on online NCBI tools to exclude any possible non-specific amplifications. The PCR cocktail and amplification condition for AMR genes were similar to those described for rDNA amplification. The amplified products were resolved in an agarose gel (2.5%). Amplified products were purified by Charge Switch PCR purification kit (Invitrogen, CA, USA) and sequenced by commercial sequencing services (Biologia Research India Pvt. Ltd.).

RESULTS AND DISCUSSION

AMR pattern of milk-borne Staphylococcus spp. and Escherichia coli: In the initial screening, the microbial growth on a selective medium with antibiotics and disc diffusion tests identified 58 isolates as positive for AMR. Two sets of universal primers specific to bacterial rDNA yielded desired amplified product size of 424 bp and 314 bp (Fig. 1).

The partial rDNA sequences of AMR-positive isolates were BLAST matched for the confirmation of the identity of bacterial genera. Among the resistant isolates, a high

Table 1. Primers for partial amplification of rDNA

Primer name	Sequence (5'-3')	Length (bp)	T_m (°C)
UNIV16SSET1FW	TCCAGACTCCTACGGGAGGCAGCAGT	26	64
UNIV16SSET1RV	GCGTGGACTACCAGGGTATCTAATCCTGT	29	63
UNIV16SSET2FW	GTTAAGTCCCGCAACGAGCGCAACC	25	63
UNIV16SSET2RV	GTGTGTACAATACCCGGGAACGTATTCACCG	31	64

Table 2. List of the primers used for partial amplification of AMR genes

Gene	Primer name	Sequence (5'-3')	Length (bp)	T_m (°C)
Quinolone group:				
aac(6')-Ib-cr	AACPFW	CGACACTTGCTGACGTACAGGAACAGTAC	29	63
	AACPRV	TGGTCTATTCCGCGTACTCCTGGATC	26	61
QNRS1P	QNRS1PFW	CAGTGTGACTTCAGCCACTGTCAGCTG	27	63
	QNRS1PRV	CCTGAACTCTATGCCAAAGCAGTTGGCAC	29	63
GyrA	GYRAFW	CTGGATTATGCGATGTCGGTCATTGTTGG	29	62
	GYRARV	GTTGCCATACCTACGGCGATACCGGA	26	63
GyrB	GYRBFW	GATAACGCTATCGACGAAGCGCTCGC	26	63
	GYRBRV	CGCCTGCGTGCAGAACGGTCATGAT	25	63
QnrB	QNRBFW	GAATATGATCACCACGCGCACCTGGTTTTG	30	63
	QNRBRV	GGCACCCATCCAACGGTTTTCCCACA	26	63
ParC	PARCFW	GTTATTGGTCAATATCATCCACATGGAG	28	57
	PARCRV	CGATACTACCATTATTACCATGCATTTC	28	56
ParE	PAREFW	GAAGCTGCACGTAAAGCTCGTGAAGA	26	60
	PARERV	ACCTTACCACGTAATGGTAATATCGCTTGG	30	60
Tetracycline group				
TET A	TETAFW	GCTTTGGGTCATTTTCGGCGAGGATC	26	61
	TETARV	TGTCGCGAAGGCAAGCAGGATGTAGC	26	63
TET B	TETBFW	GCTTCGGAAGATATCGCTAACCACTTTGG	29	62
	TETBRV	CGGCCTAAATACAGCATCCAAAGCGC	26	61
TET C	TETCFW	GCTATCAAGCTGTTTCAATAGATGAGATCTCG	32	61
	TETVRV	GATAGGCATCACTTCTTGGATAGGGATAAGG	31	62
TET D	TETDFW	CAGTCTTTACTGCTTGATGATGTGGCG	27	60
	TETDRV	GCCTCCAATTCCCATAATTTATTACGCCG	26	60
TETM	TETMFW	GTGTGACGAACTTTACCGAATCTGAACAATG	31	60
	TETRV	CGCAAAGTTCAGACTGACCTCGATGTG	27	61
TETO	TETOFW	AACTTAGGCATTCTGGCTCACGTTGAC	27	60
	TETORV	GATGTCACTGCTGTCTGGATAGTGATTCC	29	62
Methicillin group				
MECA	MECAFW	GATGGCTATCGTGTCACAATCGTTGACG	28	61
	MECARV	CTTACTGCCTAATTCGAGTGCTACTCTAGC	30	62
MECR1	MECR1FW	CTACCAACTGTCGTAGTCGAAACCATG	27	60
	MECR1RV	CGATTCACCATAACGTATATGTTCATGGCG	30	60
MECI	MECIFW	CTGCAGAATGGGAAGTTATGAATATCATTTGG	32	59
	MECIRV	GACAAGTGAATTGAAACCGCCTTTGTATAC	30	59

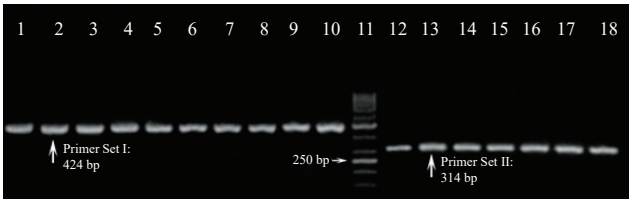

Fig. 1. Partial amplification of rDNA gene of *Staphylococcus* spp. and *E. coli* by two different sets of universal primers.

prevalence was seen for *E. coli* (63.33%), followed by *S. aureus* (28.33%), *Enterobacter* spp. (5.00%) and *Acinetobacter baumannii* (3.33%). In disc diffusion tests, a high percentage (60%) of the isolates showed resistance to two or more classes of antibiotics. Overall, we observed a high prevalence of methicillin- and tetracycline-resistant isolates (54% and 50%, respectively). Methicillin resistance was more prevalent in Gram-positive *Staphylococci* (62.96%), whereas tetracycline resistance was frequent in both Gram-positive *Staphylococci* (53.86%) and Gram-negative *E. coli* (46.14%). Among positive isolates for antibiotic resistance, the highest prevalence of resistance was shown against methicillin (90.00%), followed by tetracycline (83.33%), ofloxacin (40.00%), and ciprofloxacin (36.66%). The highest prevalence was for the dual antibiotic-resistant group (48.33%), followed by the triple antibiotic-resistant group (33.33%), multidrug (4 or more) resistant group (11.6%), and single antibiotic-resistant group (6.67%). In the dual antibiotic-resistant group, the higher prevalence was seen for oxacillin-tetracycline (33.33%), followed by oxacillin-ciprofloxacin (10.00%) and oxacillin-ofloxacin (6.64%). In the triple antibiotic-resistant group, the higher prevalence was seen for oxacillin-tetracycline-ofloxacin (30.00%), followed by tetracycline-oxacillin-ciprofloxacin (10.00%) and oxacillin-ciprofloxacin- ofloxacin (3.33%). The observed

Table 3: MIC values of randomly selected *E. coli* (EC_) and *S. aureus* (SA_) isolates against four categories of antibiotics

Isolate Id	Isolated in	MIC observed			
		Tetracycline (µg/ml)	Oxacillin (µg/ml)	Ciprofloxacin (µg/ml)	Ofloxacin (µg/ml)
SA_AC1	Cattle	64	32	-	16
SA_SU1	Buffalo	64	32	16	-
EC_CL1	Buffalo	128	32	-	-
SA_TT1	Cattle	64	-	16	-
EC_TT2	Cattle	128	16	-	-
EC_RF1	Cattle	64	64	8	-
EC_RF2	Cattle	64	32	8	-
SA_SJ1	Cattle	128	32	-	16
EC_AC7	Cattle	64	-	16	16
SA_CS1	Cattle	64	64	-	8
EC_JF1	Buffalo	32	16	-	8
SA_JF2	Buffalo	64	16	-	-
EC_JF6	Cattle	64	16	16	-
SA_SS1	Cattle	128	32	8	-
EC_SS3	Cattle	128	16	8	16
EC_KS1	Cattle	64	32	-	8
SA_KS2	Cattle	64	32	-	-
SA_NS1	Cattle	64	32	16	16
SA_NS2	Cattle	64	16	32	16
EC_NS3	Cattle	128	16	-	-
EC_NS4	Cattle	-	16	-	-
EC_KA1	Cattle	-	32	16	16
SA_KA2	Cattle	64	32	16	-
SA_KA3	Cattle	-	16	-	-
EC_KA4	Cattle	-	16	-	16
EC_KA5	Cattle	64	16	8	16
SA_KA6	Cattle	128	32	16	16
EC_YS1	Cattle	64	16	16	16
SA_BD1	Cattle	128	16	-	16

MIC Standard for Resistant as per CLSI, 2019: Tetracycline (≥16µg/ml), Oxacillin (≥0.5µg/ml), Ciprofloxacin (≥4µg/ml) and Ofloxacin (≥4µg/ml).

MIC values of 29 randomly selected isolates of resistant *E. coli* and *S. aureus* were at least 4 folds or higher than CLSI standards for tetracycline (Table 3).

Resistant *E. coli* and *S. aureus* isolates also showed high MIC (>32× CLSI standard MIC) against methicillin (oxacillin). For ciprofloxacin and ofloxacin, the observed MIC values were at least 2-4 folds higher than the standard MIC values of resistant *E. coli* and *S. aureus.*

Partial amplification of AMR genes from resistant isolates showed a fair prevalence of resistance genes against tetracycline, methicillin, and quinolones (Table 4).

Partial amplification of *Tet* genes yielded desired product size of 281 bp, 280 bp, and 358 bp for *TetD, TetO,* and *TetM*, respectively (Fig. 2a). Partial amplification of *mec* genes yielded desired product size of 224 bp, 269 bp, and 249 bp for *mecA, mecI,* and *mecR*, respectively (Fig. 2b). Partial amplification of *ParC, ParE, gyrB, gyrA,* and *qnrB* yielded 121 bp, 209 bp, 162p, 451 bp, and 12 bp products, respectively (Fig. 2c). Among *tet* genes, the abundance of *tetD* mediated resistance was highest in both *S. aureus* and *E. coli.* The abundances of *mecA, mecI,* and *mecR* were relatively high among the isolates. Percent of *E.coli* isolates resistant to methicillin had a higher abundance of the *mecA* gene, whereas *S. aureus* isolates had a relatively higher abundance of the *mecR* gene. In quinolone-resistant isolates, the abundances of *ParC* and *ParE* were higher compared to other genes. The lowest prevalence was seen for *aac* gene mediated quinolone resistance. The abundance of *mecA, mecI* and *mecR* were relatively high among the isolates. Percent of *E.coli* isolates resistant for methicillin had higher abundance of *mecA* gene, whereas *S. aureus* isolates had a relatively higher abundance of *mecR* gene. In quinolone-resistant isolates, the abundances of *ParC* and *ParE* were higher compared to other genes. The lowest prevalence was seen for *aac* gene-mediated quinolone resistance. In a similar study, antimicrobial resistance has been reported in dairy isolates from Sahiwal cattle with a high prevalence of MRSA (13.1%) and resistance against streptomycin (36.4%), oxytetracycline (33.6%), gentamicin (29.9%), and ciprofloxacin (26.2%) (Kumar *et al.* 2011). Several recent studies revealed the pan-India presence of antimicrobial resistance with high prevalence (48%) of extended-spectrum β-lactamases (ESBL) producers in Gram-negative bacilli in cow and buffalo milk and oxytetracycline (47.5%) resistance (Das *et al.* 2017). About 2.4% of *S. aureus*, isolated from the same samples were vancomycin-resistant (Bhattacharyya *et al*. 2016), while the incidence of methicillin resistance was 21.4% for *S. aureus* and 5.6% for coagulase-negative *Staphylococci* (Preethirani *et al.* 2015). Methicillin-resistant *S. aureus*, *S. epididymis* and extended-spectrum β-lactamase *E. coli* were reported from milk sampled from cows suffering from mastitis (Bandyopadhyay *et al.* 2015). ESBL-producing *E. coli* (Kar *et al*. 2015) and *K. pneumonia* (Koovapra *et al*. 2016) have also been reported in bovine milk from

Table 4. Percent of isolates positive for different AMR genes

Isolate	Tetracycline			Methicillin			
	tet D	*tet O*	*tet M*	*mec A*	*mec I*	*mec R*	
S. aureus	23.33	8.33	5.00	36.67	41.67	46.67	
E. coli	13.33	5.00	3.33	53.33	45.00	33.33	
	Quinolones						
	Aac	*Par C*	*Par E*	*qnr S*	*gyr B*	*gyr A*	*qnr B*
S. aureus	3.33	25.00	36.67	20.00	21.67	30.00	11.67
E. coli	0.00	35.00	25.00	13.33	16.67	26.67	18.33

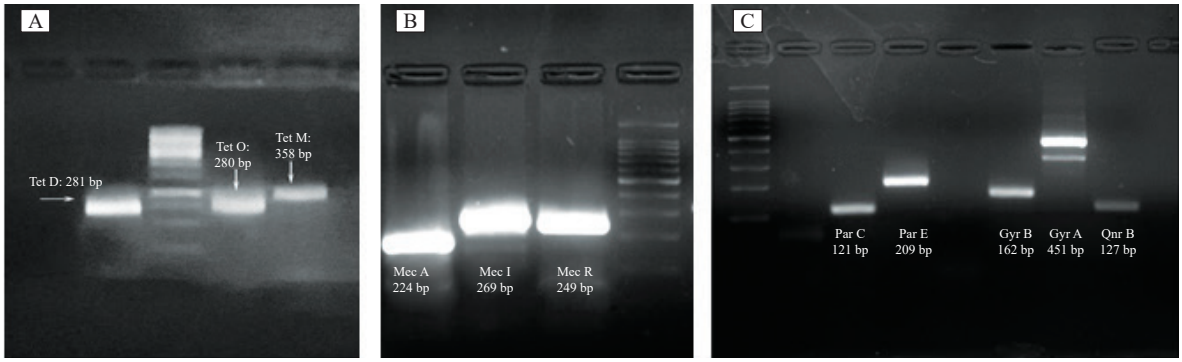

Fig. 2. Partial amplification of AMR genes from resistant isolates. (a) Tetracycline resistant genes, (b) Methicillin resistant genes and (c) quinolones resistant genes.

Odisha and three states of eastern and northeastern India. ESBL-producing *K. pneumonia* from buffalo milk has also been reported (Bandopadhyay *et al.* 2018). The majority of these isolates had harboured AmpC type β-lactamase and plasmid-mediated fluoroquinolone resistance gene(s). In our study, a high prevalence of *tetA*-mediated tetracycline resistance was observed in both *S. aureus* and *E. coli.* The abundances of *mecA, mecI,* and *mecR* were relatively high among the isolates resistant to methicillin. Over the last decades, a higher prevalence of the *mecA* gene has been detected in *S. aureus* isolates of milk origin (Gopal and Divya 2017). The *mecA* gene encodes a penicillin-binding protein (PBP2A) conferring resistance to methicillin and other β-lactam antibiotics (Liao *et al*. 2018). The higher prevalence of *mec* genes, therefore, is a major concern for both human and animal health in the region.

After analysis of milk samples from different areas of the Jammu region, we found a high prevalence of methicillin and tetracycline resistance among *E. coli* and *S. aureus* of udder origin. The tetracycline-resistance (*TetD, TetO* and *TetM*) and methicillin-resistance (*MecA, MecI* and *MecR*) genes are fairly common in *E. coli* and *S. aureus* of udder origin.

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