

ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF MULTI-DRUG RESISTANT SHIGELLA FLEXNERI, MAMMALIICOCCUS SCIURI AND ENTEROCOCCUS GALLINARUM FROM BOVINE MASTITIS AND SHIGELLOSIS CASES IN WESTERN MAHARASHTRA, INDIA

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ABSTRACT

Bovine mastitis and shigellosis-like enteric infections remain major constraints on dairy productivity and animal health, and are increasingly associated with multidrug-resistant (MDR) bacterial pathogens that threaten both veterinary therapeutics and human health via food chain. This study aimed to isolate, characterize and molecularly identify MDR bacterial pathogens from mastitis and shigellosis-suspected dairy cattle across three talukas of western Maharashtra from India including Baramati, Newasa, and Shevgaon. Twenty-five clinical samples (milk and blood) were collected from mastitis and shigellosis suspected cows, with matched samples from healthy cows as morphotype controls. Following peptone-broth enrichment and comparative spread-plating on tryptone blood agar, sixteen distinct isolates were recovered and screened for antibiotic susceptibility against 20 antibiotics using a modified Kirby–Bauer disc diffusion method. Isolates resistant to six or more antibiotics were classified as MDR. Of sixteen isolates screened, three isolates- one from shigellosis-infected milk, one from mastitis-infected milk, and one from mastitis-infected blood met MDR criteria. Biochemical profiling using the VITEK 2 system, 16S rRNA sequencing and BLASTn identified the three MDR isolates as *Shigella flexneri* (isolate MM1; 99.51% identity to strain ATCC 29903), *Mammaliicoccus sciuri* (isolate MM2; 100.00% identity to strain DSM 20345), and *Enterococcus gallinarum* (isolate B3; 99.92% identity to strain NBRC 100675).

KEYWORDS: Multidrug resistance; bovine mastitis; shigellosis; 16S rRNA gene sequencing; *Shigella flexneri*; *Mammaliicoccus sciuri*; *Enterococcus gallinarum*

1. INTRODUCTION

Bacterial infections impose a substantial burden on both human health and livestock productivity and two conditions that impose this dual impact are bovine mastitis and shigellosis. One affects the mammary gland of dairy animals and the other affects the human intestinal tract respectively. Both are caused by MDR bacterial pathogens whose treatment is being challenged by antimicrobial resistance (AMR). Studying these two diseases together highlights a shared clinical and public-health challenge. Accurate isolation and identification of these pathogens had become central to the development of possible treatment alternatives.

Mastitis is an inflammatory condition of the mammary gland that represents one of the costliest diseases in the global dairy industry, causing milk yield reduction, wastage of milk, elevated cattle maintains costs etc. 137 distinct microbial pathogens have been reported from infected milk (Shoaib et. al., 2021; Temesgen et. al., 2026) which includes *Staphylococcus aureus*, *Streptococcus agalactiae* *Mycoplasma bovis* which reside within the infected udder and spread between animals during milking, while environmental pathogens such as *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., and *Streptococcus uberis* originate from bedding, water, and manure and invade the udder opportunistically (Cheng et. al., 2020; Li et. al., 2025). Across numerous epidemiological surveys, *S. aureus* and *E. coli* consistently emerge as the two most frequently isolated agents, together with environmental *Streptococci* accounting for over 75% of clinical and subclinical cases (Belay et. al., 2022; Shoaib et. al., 2021).

Shigellosis is an acute enteric infection caused by *Shigella*, a genus of Gram-negative, non-motile, facultatively anaerobic bacilli belonging to the Enterobacterales order. The genus comprises four species *S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae* corresponding to four serogroups (A–D), with further serotype-level diversity based on lipopolysaccharide antigen variation (Samandari et al., 2025). Transmission occurs primarily via the faecal oral route, through contaminated food, water, or direct contact (Jennison et al., 2004).

Bacterial MDR was directly responsible for an estimated 1.27 million deaths in 2019 and was associated with 4.95 million deaths overall, a burden exceeding that of HIV/AIDS or malaria in the same year, and more recent modelling projects that AMR-related deaths could exceed 39 million between 2025 and 2050 without decisive intervention. *Shigella* spp. resistant to fluoroquinolones have consequently been elevated to "high priority" status on the WHO's 2024 Bacterial Priority Pathogens List, reflecting a clear upward resistance trend and a substantial diarrhoeal disease burden, particularly in resource-limited settings (Sati et al., 2025; WHO, 2024), and MDR *Shigella* strains with reduced susceptibility to fluoroquinolones, azithromycin, and third-generation cephalosporins have already been documented across multiple continents, leaving clinicians with a shrinking arsenal of effective treatment options (Baker et al., 2023). A parallel trend defines mastitis pathogens: longitudinal dairy-herd surveillance has recorded *E. coli* isolates with multidrug resistance rates as high as 45.86%, alongside rising resistance in *S. aureus* and *Klebsiella* spp. isolates, with some Multiple Antibiotic Resistance indices approaching 1.0 (Janus et al., 2024; Moradi et al., 2025), while methicillin-resistant *S. aureus* (MRSA) and extended-spectrum β -lactamase (ESBL)-producing *E. coli* and *Klebsiella* have also been recovered from bovine milk samples, showing that resistance mechanisms of major clinical concern are already established within veterinary reservoirs (Saini et al., 2012).

These findings make isolation and identification of MDR bacterial pathogens from mastitis and shigellosis cases so critical not only for diagnostic, but as a prerequisite for meaningful therapeutic advancement. Antibiotic therapy without knowledge of the causative organism's resistance profile risks both treatment failure and resistance development. Hence reliable isolation and phenotypic/genotypic characterization through culture-based identification, antibiotic susceptibility testing, and molecular detection of resistance determinants allows clinicians and researchers to distinguish susceptible from resistant strains, tailor antimicrobial regimens to individual cases, and track the emergence of resistance mechanisms (Janus et al., 2024).

The present study was therefore undertaken to isolate, phenotypically and biochemically characterize, and molecularly identify *S. flexneri*, *M. sciuri*, and *E. gallinarum* recovered from bovine mastitis- and shigellosis-associated milk and blood samples in Western Maharashtra, India, and to profile their multidrug-resistance patterns using standard antibiotic susceptibility testing. By establishing a robust, confirmed-MDR isolate panel, this work aims to lay the diagnostic and epidemiological foundation for downstream translational efforts including the evaluation of alternative antimicrobial strategies, such as insect-derived antimicrobial peptides, against these emerging and under-characterized veterinary pathogens.

2. MATERIALS AND METHODS

2.1 Physiological examination of cattles

The study was conducted on dairy cattle maintained on farms by businessmen, individual farmers and milk vendors in and around Baramati, Newasa, and Shevgaon tehsil of Maharashtra, India. Cows were examined individually in their housing environment for the udder, teats, and general physical conditions for detection of bovine mastitis and shigellosis. The information on recent health status, milk production, and gastrointestinal history was obtained from owners and farm records to assign animals status of mastitis or shigellosis suspected categories (Sohidullah et al., 2016).

Cows with a documented history of prior antibiotic treatment, together with those showing overt clinical signs, were preferentially selected to enhance the probability of recovering MDR strains. Matched milk and blood samples from apparently healthy cows were collected in parallel as control.

2.2 Collection of Samples

A total of 25 clinical samples were collected from selected sites. Fourteen from mastitis-suspected cows (ten milk, four blood) and eleven from shigellosis-suspected cows (five milk, six blood). All samples were transported to the laboratory under controlled conditions that preserved sample integrity for downstream microbiological processing (Bakr et al., 2019).

2.3 Enrichment of microorganisms

For the enrichment purpose, one mL of each collected clinical sample was aseptically inoculated into 50 mL of sterile peptone broth to facilitate recovery and proliferation of bacteria (Cervinkova et al., 2013). Inoculated broths were incubated at 37 °C on a rotary shaker for 4 days to allow injured, stressed, or slow-growing cells to recover and reach detectable densities.

2.4 Isolation of pathogens

Serial ten-fold dilutions of each enriched sample were prepared in sterile normal saline (up to 10^{-10}), and 0.1 mL aliquots of the 10^{-4} , 10^{-5} , and 10^{-6} dilutions were spread onto sterile tryptone blood agar supplemented with 5% calf blood. Milk and blood samples from apparently healthy cows were processed identically and plated in parallel to serve as morphotype controls. All plates were incubated at 37 °C for 24 h. Colonies exhibiting morphology distinct from that on control plates (differences in size, shape, hemolysis pattern, pigmentation, or surface characteristics) were selected and purified by iterative subculture on fresh tryptone blood agar. This comparative spread-plate strategy allowed subtle differences in colony morphology and hemolysis to be recognized against the background of the normal udder/blood microbiota. Each

purified isolate was assigned an identification code based on sample origin and colony morphology and maintained on agar slants at 4°C for further characterization.

2.5 Morphological characterization

Colony characteristics (size, shape, color, margin, elevation, opacity, consistency) were recorded on tryptose blood agar. Gram staining, spore staining, and motility testing were performed using standard microbiological procedures to obtain a preliminary phenotypic classification prior to biochemical and molecular identification (Ganesh et. al., 2024).

2.6 Antimicrobial susceptibility testing and MDR screening

Antibiotic susceptibility of 16 screened isolates was determined by the disc diffusion method on Mueller Hinton Agar (MHA) using a modified Kirby–Bauer technique (Bauer et. al., 1966). HiMedia antibiotic polydiscs with 20 antibiotics comprising cephalothin (CEP), clindamycin (CD), co-trimoxazole (COT), erythromycin (E), gentamicin (GEN), ofloxacin (OF), penicillin-G (P), vancomycin (VA), ampicillin (AMP), chloramphenicol (C), oxacillin (OX), linezolid (LZ), azithromycin (AZM), amikacin (AK), clarithromycin (CLR), teicoplanin (TEI), methicillin (MET), amoxycylav (AMC), novobiocin (NV), and tetracycline (TE) were used. A standardized inoculum of each isolate was prepared to match the 0.5 McFarland turbidity standard ($\sim 1 \times 10^8$ CFU/mL) and evenly swabbed onto MHA plates, which were allowed to dry before disc placement. Plates were subjected to a 30 min pre-diffusion step at 4°C followed by incubation at 37°C for 24 h. Zones of inhibition were measured and interpreted as susceptible, intermediate, or resistant according to standard CLSI breakpoint criteria (CLSI, 2023). Isolates resistant to six or more of the antibiotics were classified as multidrug-resistant (MDR) and selected for further characterization (Cervinkova et. al., 2013).

2.7 Biochemical characterization (API profiling)

Biochemical characterization of the three MDR isolates was performed using the VITEK 2 automated system (bioMérieux), employing the Gram-negative identification card for MM1 isolate and the Gram-positive identification card for isolate MM2 and B3. Each card evaluated 43 biochemical reactions, including carbohydrate utilization, enzyme activities, arginine dihydrolase systems, growth in 6.5% NaCl, and susceptibility to novobiocin, bacitracin, optochin, and the vibriostatic agent O/129, complementing the molecular identification (Maina et. al., 2021).

2.8 Molecular identification

Colony lysates were prepared by suspending one to two well-isolated colonies from quality-controlled plates in 50 µL molecular-biology-grade water followed by heating at 95°C for 10 min, and centrifugation (Loberiza et. al., 2025), the supernatant used directly as PCR template. The near-full-length 16S rRNA gene was amplified using universal primers 16S27F (5'-CCAGAGTTTGATCMTGGCTCAG-3') and 16S1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Weisburg et. al., 1991) with a commercial PCR master mix under standard denaturation, annealing and extension conditions. The 1500 bp product was confirmed by agarose gel electrophoresis. Amplicons were purified by salt precipitation cleanup and subjected to bidirectional Sanger sequencing using BigDye Terminator v3.1 chemistry on an ABI 3500XL Genetic Analyzer (Sanger et. al., 1977). The chromatograms were quality-trimmed at both termini and overlapping forward, reverse, and internal primer reads were assembled into consensus contigs ($\geq 1,400$ bp) per isolate. Consensus sequences were queried by BLASTn against the NCBI 16S rRNA reference database (Altschul et. al., 1990), with species-level identification assigned at $\geq 99\%$ identity with full query coverage against a type-strain sequence and genus-level identification at $\geq 97\%$; isolates without a single dominant match were reported as most closely related to the relevant taxon. Representative reference sequences were retrieved from NCBI and aligned with isolate consensus sequences to construct maximum-likelihood phylogenetic trees in IQ-TREE v2.1.2 under the Kimura two-parameter model with 1,000 bootstrap replicates (Tamura et. al., 2013).

2.9. Sequence Submission in NCBI

Confirmed 16S rRNA gene sequences of *Shigella flexneri*, *Mammaliicoccus sciuri*, and *Enterococcus gallinarum* were deposited in the NCBI GenBank nucleotide database using the BankIt web-based submission tool. Each consensus FASTA sequence was submitted individually under "Individual sequence" type, with submitter details, molecule type, BLAST-confirmed organism name, source information, collection date and the 16S ribosomal RNA gene feature annotated. Following automated validation and correction of any formatting errors, the finalized submissions were released, and unique GenBank accession numbers were issued for each isolate.

3. RESULTS

3.1 Physiological examination and sample collection

Clinical examination successfully classified cows into mastitis-suspected and shigellosis-suspected groups on the basis of udder signs (inflammation, pain, heat and milk abnormalities) and systemic/enteric signs (reduced yield, weight loss, diarrhea). Cows classified as mastitis-suspected exhibited one or more cardinal signs of udder inflammation and altered viscosity and clots in milk. The shigellosis-suspected cows showed reduced milk yield, weight loss, poor body condition, diminished feed intake and episodes of diarrhea.

A total of 25 clinical samples were collected, 14 from mastitis-suspected cows (10 milk, 4 blood) and 11 from shigellosis-suspected cows (5 milk, 6 blood) from Baramati, Newasa, and Shevgaon .

3.2 Enrichment, Isolation and Morphological characterization

Enrichment of all 25 samples were carried out in sterile peptone broth to facilitate recovery and proliferation of selected bacteria. After incubation high growth of bacterial pathogens observed in flask.

Comparative spread-plating of enriched samples against healthy-cow controls on tryptone blood agar yielded sixteen distinct bacterial isolates; nine from mastitis-infected milk, one from shigellosis-infected milk, three from mastitis-infected blood, and three from shigellosis-infected blood. Colony morphology, Gram reaction, spore staining, and motility testing revealed a mixed population of Gram-positive and Gram-negative organisms with coccal and rod-shaped morphologies, some Gram-positive rods forming endospores, and variable motility providing a preliminary phenotypic basis for the subsequent biochemical and molecular work.

Table 1. Morphological Characterization of the screened 16 isolates enriched in peptone broth

Isolates	Size (mm)	Shape	Color	Margin	Elevation	Opacity	Consistency	Gram nature	Motility	Spore formation
MM1	3	Circular	White	Entire	Convex	Translucent	Smooth	Gram negative rod	Non motile	Non-Spore forming
MM2	2	Circular	white	Entire	Convex	Opaque	Smooth	Gram positive cocci	Non motile	Non-Spore forming
MM3	1	Circular	white	Entire	Convex	Opaque	Smooth	Gram positive cocci	Non motile	Non-Spore forming
SM1	1	Circular	Grey	Entire	Convex	Opaque	Smooth	Gram positive cocci	Non motile	Non-Spore forming
SM2	2	Circular	White	Entire	Convex	Opaque	Smooth	Gram positive cocci	Motile	Non-Spore forming
SM3	4	Circular	Grey	Entire	flat	Opaque	Smooth	Gram negative rod	Non motile	Non-Spore forming
NM1	2	Circular	Yellow	Entire	Convex	Opaque	Smooth	Gram positive rod	Non motile	Non-Spore forming
NM2	3	Circular	White	Entire	flat	Opaque	Smooth	Gram positive cocci	Non motile	Non-Spore forming
NM3	2	Circular	Yellow	Entire	Convex	Opaque	Smooth	Gram negative rod	Non motile	Non-Spore forming
GM1	2	Circular	White	Entire	Convex	Opaque	Smooth	Gram negative rod	Non motile	Non-Spore forming
B1	2	Circular	white	Entire	Convex	Opaque	Smooth	Gram negative rod	Non motile	Non-Spore forming
B2	1	Circular	White	Entire	Convex	Translucent	Smooth	Gram positive cocci	Non motile	Non-Spore forming
B3	2	Circular	White	Entire	Slightly convex	Opaque	Smooth	Gram positive cocci	Motile	Non-Spore forming
B4	1	Circular	White	Entire	Convex	Transparent	Smooth	Gram negative rod	Non motile	Non-Spore forming
B5	2	Circular	Yellow	Entire	Convex	Opaque	Smooth	Gram positive cocci	Non motile	Non-Spore forming
B6	2	Circular	white	Entire	Convex	Opaque	Smooth	Gram positive cocci	Non motile	Non-Spore forming

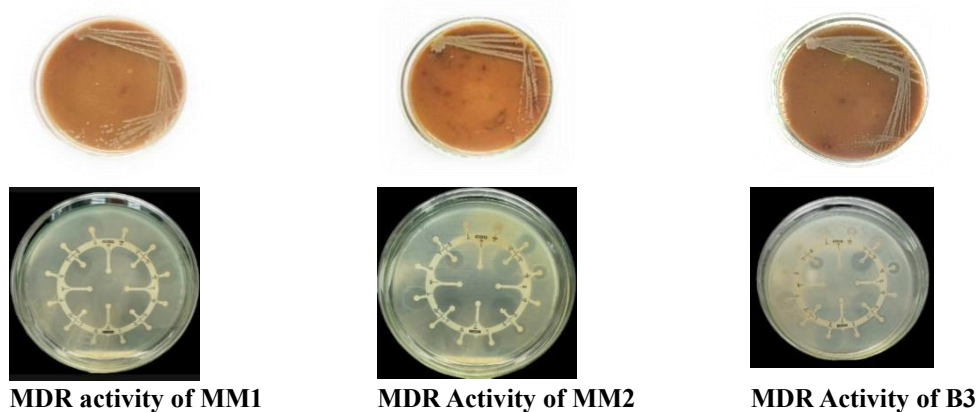


Figure 1 : Isolation of veterinary pathogens

Table 2: A report of MDR characterization of the isolates

Sr · No	Antibiotics	Sym bol	M M 1	M M 2	M M 3	SM 1	S M 2	SM 3	N M 1	N M 2	N M 3	G M1	B1	B2	B 3	B4	B5	B6
1	Cephalothi n	CEP	-	31	8	-	2 3	-	20	15	31	23	19	-	-	-	31	23
2	Clindamyci n	CD	-	20	-	-	8	-	-	-	22	-	33	10	-	25	23	-
3	Co- trimoxazole	COT	20	26	22	30	2 4	14	32	19	16	20	29	33	33	20	30	17
4	Erythromyc in	E	10	13	20	20	4	20	10	35	30	10	13	25	19	20	30	18
5	Gentamyci n	GEN	29	-	28	30	2 8	25	28	33	28	28	35	25	24	20	28	32
6	Ofloxacin	OF	25	26	24	23	2 4	22	25	20	23	23	27	20	20	21	21	22
7	Penicillin- G	P	-	13	12	15	7	11	10	29	34	14	23	14	12	10	35	25
8	Vancomyci n	VA	17	-	9	15	1 0	14	13	-	19	8	23	10	-	11	21	22
9	Ampicillin	AMP	-	18	15	11	-	14	-	21	32	-	20	12	-	12	35	22
10	Chloramph enicol	C	28	-	12	12	2 3	22	20	22	25	23	13	-	8	14	26	25
11	Oxacillin	OX	-	8	-	8	8	13	-	14	17	-	-	8	-	-	20	15
12	Linezolid	LZ	-	-	-	-	-	-	-	-	30	-	35	7	-	-	30	3
13	Azithromyc in	AZM	-	-	23	24	1 5	23	15	28	22	20	26	24	-	19	25	24
14	Amikacin	AK	34	27	35	30	3 0	28	33	32	29	30	37	30	30	24	30	33
15	Clarithromy cin	CLR	20	20	20	20	1 1	25	9	30	35	10	33	23	15	20	30	25
16	Teicoplanin	TEI	-	9	8	10	8	-	-	18	17	8	22	-	-	8	17	18
17	Methicillin	MET	-	-	-	8	8	-	7	13	10	-	10	-	-	-	12	9
18	Amoxyclav	AMC	25	15	10	14	1 8	12	18	15	33	13	23	13	15	13	35	21
19	Novobiocin	NV	-	14	10	12	1 1	12	14	15	17	10	24	12	13	11	16	24
20	Tetracyclin e	TE	20	21	20	15	1 9	12	20	20	21	15	20	17	17	17	23	20

3.4 Biochemical characterization

VITEK 2 profiling using the Gram-negative card for MM1 and the Gram-positive card for MM2 and B3 generated 43-parameter biochemical fingerprints for each isolate, encompassing carbohydrate utilization, enzymatic activities, arginine dihydrolase activity, growth in 6.5% NaCl, and susceptibility/resistance to novobiocin, the vibriostatic agent O/129, bacitracin, and optochin as appropriate to each card type. These automated biochemical profiles were consistent with the molecular identifications

Table 3: Biochemical profiling of the isolates (A): Tests for isolate MM1; (B): Tests for isolates MM2 and B3

Sr. No	Test	MM1 Result	Sr. No	Test	MM1 Result	Sr. No	Test	MM1 Result	Sr. No	Test	MM1 Result
1	APPA	-	12	ADO	-	23	PyrA	-	34	IARL	-
2	H2S	-	13	BNAG	-	24	AGLTp	-	35	dGLU	+
3	BGLU	-	14	dMAL	+	25	dMAN	+	36	dMNE	+
4	ProA	-	15	LIP	-	26	PLE	-	37	TyrA	+
5	SAC	-	16	dTAG	-	27	dTRE	+	38	CMT	+
6	ILATk	-	17	AGLU	-	28	SUCT	+	39	NAGA	-
7	GlyA	-	18	ODC	+	29	LDC	+	40	IHISa	-
8	O129R	-	19	GGAA	-	30	IMLTa	-	41	ELLM	-
9	dCEL	-	20	BXYL	-	31	dsOR	+	42	5KG	-
10	GGT	-	21	URE	-	32	MNT	-	43	AGAL	-
11	PHOS	+	22	BGUR	-	33	BGAL	+	44	OFF	+
									45	BAlap	-
A											

Sr · No	Test	Result		Sr · No	Test	Result		Sr · No	Test	Result		Sr · No	Test	Result	
		MM 2	B 3			MM 2	B 3			MM 2	B 3			MM 2	B 3
1	AMY	-	-	12	OPTO	-	-	23	O129R	+	+	33	SAL	+	+
2	APPA	-	-	13	PIPLC	-	-	24	dXYL	-	-	34	ADH1	+	-
3	LeuA	-	-	14	CDEX	-	-	25	AspA	-	-	35	BGAR	-	-
4	AlaA	-	-	15	ProA	-	-	26	BGUR r	-	-	36	AGAL	-	-
5	dRIB	-	-	16	TyrA	-	-	27	dSOR	+	+	37	URE	-	-
6	NOVO	-	-	17	ILAT k	+	-	28	LAC	-	-	38	BGAL	-	-
7	dRAF	-	-	18	NC6.5	+	+	29	dMAN	+	+	39	AMA N	-	-
8	PyrA	-	-	19	dGAL	+	-	30	MBdG	+	+	40	PHOS	+	+
9	POLY B	-	-	20	dMAL	+	+	31	AGLU	+	-	41	BACI	+	+
10	PUL	-	-	21	dMNE	+	+	32	SAC	+	+	42	dTRE	+	+
11	ADH2s	-	-	22	NAG	+	-								
B															

3.5. Molecular identification

PCR amplification of the 16S rRNA gene from isolates MM1, MM2, and B3 using the universal primers 16S27F/16S1492R consistently produced single, distinct ~1.5 kb bands with no visible non-specific amplification or primer-dimer formation. Bidirectional Sanger sequencing on an ABI 3500XL Genetic Analyzer generated chromatograms that yielded consensus 16S rRNA sequences of sufficient length and quality after trimming and contig assembly for reliable taxonomic analysis (sequence IDs: 25E110_220_MM1, 25E110_221_MM2, 25K110_098_B3).

BLASTn analysis (Table 3) showed that isolate MM1 aligned most closely with *Shigella flexneri* strain ATCC 29903 (NR_026331.1; 100% query coverage, 99.513% identity), with several other Enterobacteriaceae (*S. boydii*, *S. sonnei*, *S.*

dysenteriae, *Escherichia fergusonii*, *E. coli*, *E. albertii*) also scoring highly ($\geq 98.3\%$ identity), reflecting the well-known conservation of 16S rRNA within this family. Isolate MM2 matched *Mammaliicoccus sciuri* strain DSM 20345 (NR_025520.1) with 100% coverage and 100.000% identity, clearly separating from *Staphylococcus* spp. (identities $\leq 97.5\%$). Isolate B3 matched *Enterococcus gallinarum* strain NBRC 100675 (NR_113924.1) with 100% coverage and 99.920% identity, the highest bit score among all *Enterococcus* comparators tested. All the Maximum-likelihood phylogenetic tree was successfully generated.

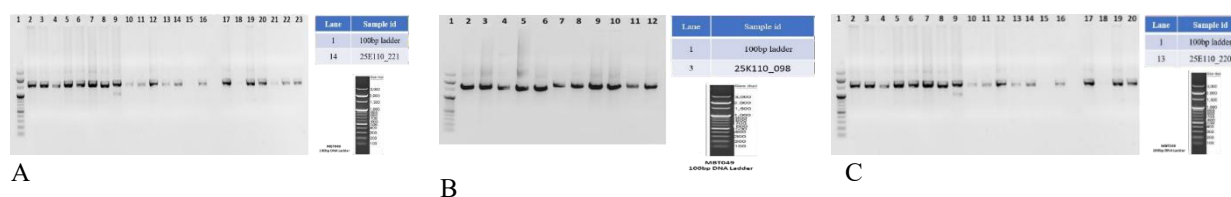
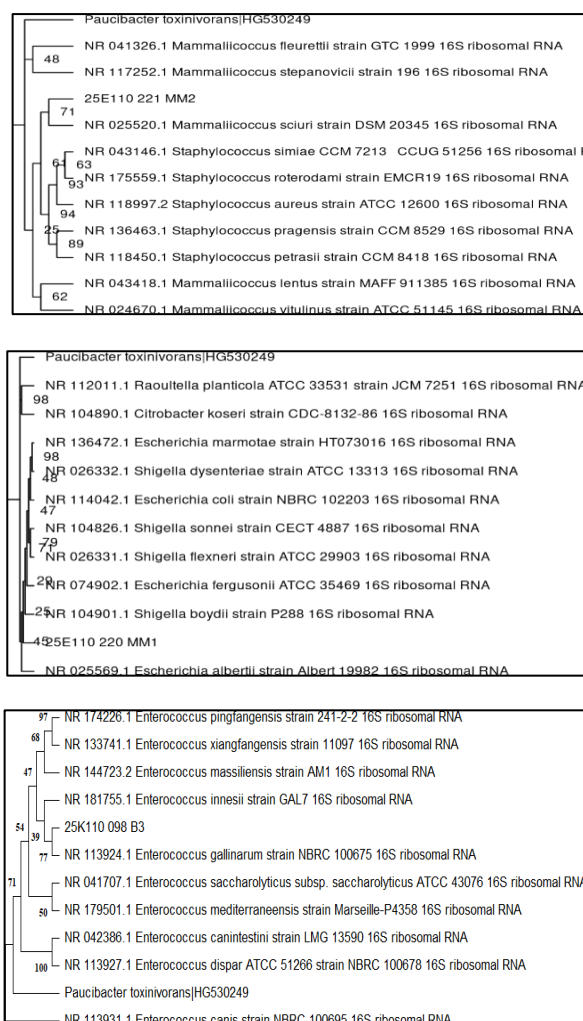


Figure 2. Agarose gel electrophoresis of 16S rRNA gene PCR amplicons (~1,500 bp) for isolates (A) MM1 (*Shigella flexneri*, sample 25E110_220), (B) MM2 (*Mammaliicoccus sciuri*, sample 25E110_221), and (C) B3 (*Enterococcus gallinarum*, sample 25K110_098). Lane 1 in each gel = 100 bp DNA ladder; sample lane indicated in the accompanying key.

Figure 3. Maximum-likelihood phylogenetic trees (IQ-TREE v2.1.2, Kimura substitution model, 1,000 bootstrap replicates) based on 16S rRNA gene sequences, showing the phylogenetic placement of (A) isolate MM1 within the *Shigella flexneri* clade, (B) isolate MM2 within the *Mammaliicoccus sciuri* clade, and (C) isolate B3 within the *Enterococcus gallinarum* clade, relative to closest type/reference strains identified by BLASTn. Numbers at nodes indicate bootstrap support (%).



3.7. Sequence Submission in NCBI

The 16S rRNA gene sequences of the three multidrug-resistant isolates MM1, MM2 and B3 were successfully deposited in the NCBI GenBank nucleotide database and assigned unique accession numbers PX795123, PX795133 and PX795141 respectively, confirming their public retrievability. Also providing definitive, citable molecular confirmation of the isolates' taxonomic identities.

4. DISCUSSION

The clinical presentations observed in this study, including udder inflammation and abnormal milk consistency alongside systemic signs such as reduced yield, weight loss, and diarrhea, align with standard veterinary diagnostic criteria (Radostits

et. al., 2007). This reinforces field-based examination as a reliable preliminary screening tool prior to laboratory confirmation (Sohidullah et. al., 2016). The data supported the microbiological procedures, when non-selective peptone broth enrichment and comparative spread-plating against healthy-cow controls yielded pathogen-associated isolates showing varied morphological characteristics, consistent with standard phenotypic grouping schemes (Bergey et. al., 2000; Cappuccino et. al., 2014). Also, Automated VITEK 2 profiling, relying on bacitracin/optochin differentiation for Gram-positive isolates (Facklam et. al., 1974) and enzymatic/carbohydrate patterns for the Gram-negative isolate (Gavin et. al., 2002), showed strong concordance with molecular identifications, reinforcing its diagnostic reliability (Gavin et. al., 2002). Resistance to β -lactams and macrolides alongside susceptibility to gentamicin, amikacin, linezolid, and vancomycin is in consistence with widely reported resistance trends in mastitis and shigellosis pathogens (Ceniti et. al., 2017). The three isolates (MM1, MM2, B3) met the internationally accepted MDR criterion of non-susceptibility to agents across two or more antimicrobial categories (Magiorakos et. al., 2012). 16S rRNA amplification using the universal 27F/1492R primers (Weisburg et. al., 1991) confirmed MM1 as *Shigella flexneri* despite known *Escherichia-Shigella* resolution limitations (Ragupathi et. al., 2017), while MM2 and B3 were identified as *Mammaliicoccus sciuri* (Madhaiyan et. al., 2020) and *Enterococcus gallinarum* respectively. Public accessioning of these sequences further strengthens reproducibility and credibility of the identifications (Janda et. al., 2007; Benson et. al., 2013).

5. CONCLUSION

This study successfully isolated, phenotypically screened, and molecularly identified three multidrug-resistant bacterial pathogens of *Shigella flexneri*, *Mammaliicoccus sciuri*, and *Enterococcus gallinarum* from mastitis and shigellosis-suspected dairy cattle in Baramati, Newasa, and Shevgaon, Maharashtra, India. All three isolates showed resistance to six or more of twenty tested antibiotics, with a shared pattern of β -lactam and macrolide resistance. These findings reinforce the need for continued antimicrobial resistance surveillance in regional dairy production systems and highlight the urgency of developing alternative antimicrobial strategies for MDR veterinary pathogens. The pathogens are confirmed by biochemical profiling, 16S rRNA gene sequencing, BLASTn, and maximum-likelihood phylogenetics provides a defined and taxonomically validated target set for ongoing and future evaluation of new drugs against multidrug-resistant veterinary pathogens.

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