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RESEARCH ARTICLE

Prevalence of *Enterococcus faecalis* and *Pseudomonas aeruginosa* from mastitis milk and their antimicrobial resistance

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Abstract: Mastitis an inflammatory condition of mammary glands is a multi-factorial disease of dairy animals. It is characterized by physical, chemical and bacteriological changes in the milk. In the present study prevalence of *Enterococcus faecalis* (*E. faecalis*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were studied along with their antibiotic resistance pattern. One hundred and ten milk samples were screened from mastitis cattle and buffaloes in and around Ludhiana from December 2020 till June 2021. The prevalence of *E. faecalis* and *P. aeruginosa* was 7.05% and 4.70% respectively. The isolates of *E. faecalis* were resistant to penicillin G (100%), vancomycin, erythromycin, and tetracycline (83.33%) and sensitive to nalidixic acid (100%), streptomycin (83.33%), oxacillin, gentamicin, ciprofloxacin and ceftriaxone (66.66%) whereas isolates of *P. aeruginosa* were resistant to ampicillin, ciprofloxacin, ceftriaxone, penicillin G, streptomycin, amikacin, vancomycin (100%), tetracycline and teicoplanin (75%) and sensitive to cefuroxime, gentamicin, and oxacillin (100%). In *E. faecalis, vanA, vanB* (50%), *tetL* (83.33%) and *mrsA/B* (100%) antibiotic genes were amplified whereas in *P. aeruginosa*, *aadA, DHAM* (100%), *sulI, sulII* (100%), *gyrA, gyrB* (100%) and *tetC* (50%) antibiotic genes were amplified. Thus, it is concluded that there is an evolving prevalence of environmental pathogens such as *E. faecalis* and *P. aeruginosa* in mastitis which is alarming and thus necessary action and plans should be followed for controlling the antibiotic resistance in these pathogens as these are of zoonotic significance.

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Introduction

Mastitis is a multifactorial complex disease characterized by inflammation of the parenchyma of the mammary gland, as demonstrated by physical, chemical, and bacteriological changes in milk, as well as pathological abnormalities in glandular tissues (Constable et al. 2017). It has mainly two forms clinical and subclinical. In most dairy herds, subclinical mastitis is 3 to 40 times more frequent than the clinical mastitis and overall it generates the biggest losses to the farmers (Bachaya et al. 2011, Singh et al. 2018). *Enterococcus* spp. and *Pseudomonas aeruginosa* are one of the important environmental pathogens causing mastitis. *Enterococcus* spp. is an environmental causative agent of mastitis and seen in the gut flora of healthy humans and animals. *Enterococcus* spp. coexisted since long but has been highlighted in recent years causing diseases like bacteraemia, endocarditis, meningitis, urinary tract infections, soft tissue infections and bovine mastitis. There are many species of *Enterococcus* such as *E. fecalis*, *E. faecium*, *E. durans*, *E. avium*, *and E. gallinarum*. Among these species *E. faecalis* (80%) and *E. faecium* (10%-15%) are the most commonly found bacteria that cause mastitis (Różańska et al. 2019). Enterococci are important because of their ability to harbour antimicrobial resistance genes (Klare et al. 2003). *Enterococcus* spp. are known to as indicator organisms for antimicrobial resistance development (Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP, 2003) as they provide accurate information on the animals prior antibiotic treatment (Centre for Disease Control and Prevention) (CDC, 2002).

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram negative bacterium, ubiquitous, and has a wide host range with metabolic versatility. They multiply rapidly in various environmental conditions and milk being one of them. *P. aeruginosa* has been associated with sub-clinical mastitis. The isolates of *P. aeruginosa* are strong biofilm producer which decreases the potency of antibiotics and leads to chronic mastitis (Melchior et al. 2006). In human and veterinary medicine,

antibiotic resistance is a global problem and it generally occurs due to extensive use of antibiotics. Bovine mastitis is the single most common cause for antimicrobial use in lactating cattle worldwide and there is a variety of antimicrobials that are used to prevent for the cure of mastitis. The antimicrobial therapy for enterococci infections is complicated because of the inherent resistance exhibited. *P. aeruginosa* too poses a serious therapeutic challenge for treatment because of its ability to develop resistance to multiple classes of antibacterial agents quickly (Lambert, 2002).Thus, identification of the mastitis-causing pathogens along with their antibiotic resistance assumes great importance for the effective control of mastitis. Therefore, the present study was planned with an objective to isolate and identify *Enterococcus* spp. and *Pseudomonas* spp. from mastitis milk along with their antimicrobial resistance pattern for the purpose of gaining a better understanding of the importance of this infection in dairy animals.

Material and methods

Sample collection

A total of 110 milk samples were collected from mastitis animals from Teaching Veterinary Clinical Complex (TVCC) GADVASU (66), Haibowal dairies (25), Noor Mahal farm (7), and from an organized dairy farm (12) at Ludhiana. All the samples were tested by SLS test for initial screening and all those positive were transferred immediately to laboratory on ice for the isolation of bacteria.

Isolation and identification of bacteria

The milk samples brought to the laboratory were thoroughly mixed and inoculated on basal media like Brain Heart Infusion (BHI) Agar and Nutrient Agar. These plates were incubated at 37°C for 12-24 h to observe the bacterial growth. The bacteria from both the media were further inoculated on bile esculin agar, enterococcus agar base and cetrimide agar. *Enterococococcus* isolates on bile esculin agar produced brown colonies with black discolouration due to hydrolysis of esculin and they tolereated 6.5% NaCl concentration. *Pseudomonas* isolates produced pyocyanin and pyoverdin on cetrimide agar. Further, they were subjected to biochemical tests like indole test, methyl red test, voges proskauer's test, citrate utilization, oxidase test, catalase tests, urease, nitrate reduction, esculin hydrolysis, 6.5% NaCl tolerance, triple sugar iron test, fermentation of various sugars viz. glucose, lactose, sucrose, sorbitol and maltose for confirmation. All the isolates were confirmed by MALDI-TOF (Bruker daltonics, GmBH).

Confirmation of *E. faecalis* strains

These isolates when grow on bile esculin gives brown coloured colonies with black discolouration due to hydrolysis of esculin and they can tolerate bile salts. They have ability to grow on 6.5% NaCl concentration (Table 1).

Confirmation of *P. aeruginosa* strains

These isolates produce pigments such as pyocyanin and pyoverdin which was examined using the "fluorescent technique," which involved growing the isolates on cetrimide and then exposing them to UV light illumination (Table 2).

Antibiotic Sensitivity Testing

All the isolates were tested for antibiotic sensitivity using Bauer et al. (1966) disc's diffusion method using twenty one antibiotics viz., amikacin (30 mcg), amoxycillin (10 mcg), ampicillin (10 mcg), ampicillin/sulbactam (10/10 mcg), cefoperazone (75 mcg), ceftriaxone (30 mcg), cefuroxime (30 mcg), cephalothin (30 mcg), ciprofloxacin (5 mcg), co-trimoxazole (25 mcg), enrofloxacin (10 mcg), erythromycin (15 mcg), gentamicin (10 mcg), nalidixic acid (30 mcg), oxacillin (1 mcg), penicillin G (10 mcg), sparfloxacin (5 mcg), streptomycin (10 mcg), teicoplanin (30 mcg), tetracycline (30 mcg) and vancomycin (30 mcg). In brief, the individual bacterium were grown overnight (10-12 h) in BHI broth at 37°C and was spread uniformly on Muller Hinton Agar with the help of sterilized cotton swab. Antibiotic discs were placed equidistantly under sterile conditions and the plates were then incubated for 12-24 h at 37°C. The zones of sensitivity was measured and were classified as sensitive, intermediate or resistant on the basis of zone of inhibition as per the standard guidelines of CLSI Standards (CLSI, 2018) (Table 3).

DNA Extraction

The DNA of *Enterococcus* spp. and *P. aeruginosa* isolates was extracted using NucleoSpin® Microbial DNA kit as per the manufacturer's instructions.

Polymerase Chain Reaction

PCRs were carried out for *Enterococcus* spp. and *Pseudomonas* spp. using genus specific primers (Table 4). A 25 µl PCR reaction mixture was formulated using 12.5 µl of master mix (2X Go Taq Green Master mix, (Promega, WI USA), 0.5 µl of 20 pmol/ul of each forward and reverse primers (IDT, USA), 1.0 µl of template DNA and finally the reaction volume was made up to 25 µl using NFW (NEB Labs, USA). PCR was performed using thermocycler (Veriti, Applied Biosystem, USA) with the following conditions; an initial denaturation at 94°C for 2 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes for *Enterococcus* spp. and initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 1 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes for *P. aeruginosa*.

Detection of antibiotic resistance genes in *E. faecalis*

All the *E. faecalis* isolates were tested for the presence of *vanA, vanB, vanC1, tetL, tetk* and *msrA/B* (Table 5). A 25µl reaction mixture was formulated by using 12.5µl (2X Go Taq Green Master Mix) (Promega, WI USA), 1µl of 20 pmol/ul of each forward and reverse primers for each of the antibiotic resistant genes (IDT, USA), 2µl of template DNA and 8.5µl of nuclease free water. PCR was performed using thermocycler (Veriti, Applied Biosystem, USA). For *vanA* and *vanB* genes, initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation at 94°C for1 minute, annealing at 54°C for 1 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 7 minutes. For *vanC1* gene, an initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes. For *tetK, tetL* genes, an initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 60 seconds, annealing at 50°C for 60 seconds and extension at 72°C for 1 minute 30 seconds followed by a final extension 72°C for 5 minutes. For *msrA/B* gene, an initial denaturation at 94C for 3 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 120 seconds and extension at 72°C for 1 minute 30 seconds followed by a final extension at 72°C for 10 minutes.

Detection of antibiotic resistance genes in *P. aeruginosa*

All the *P. aeruginosa* isolates were tested for the presence of *blaTEM, blaSHV, sulI, sulII, aadA, DHAM, MOXM, tetA, tetB, tetC, oxa-1, blaCTX-M, gyrA* and *gyrB* (Table 6). A 25µl reaction mixture by adding 12.5µl master mix (2X Go Green Taq Master Mix) (Promega, WI USA), 1µl of 20 pmol/ul of each forward and reverse primers for each of the antibiotic resistant genes (IDT, USA), 2µl of template DNA and 8.5µl of nuclease free water. PCR was performed on a thermocycler (Veriti, Applied Biosystem, USA). For *blaTEM, blaSHV, Sul1, SulII* and *tetC* genes an initial denaturation at 94°C for 5minutes, 30 cycles 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1.5 minutes followed by a final extension at 72°C for 10 minutes. For *tetA, tetB, DHAM, MOXM* and *aadA* an initial denaturation at 95°C for 5minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes. For *blaCTX-M,* an initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 95°C for 20 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 30 seconds followed by a final extension at 72°C for 3 minutes. For *oxa-1*, an initial denaturation at 96°C for 5 minutes, 35 cycles of denaturation at 96°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes. For *gyr*A, an initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 45 seconds, annealing for 57°C for 45 seconds, extension at 72°C for 45 seconds, followed by a final extension at 72°C for 5 minutes. For *gyr*B, an initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 45 seconds, annealing for 40°C for 45 seconds, extension at 72°C for 45 seconds followed by a final extension at 72°C for 5 minutes.

Gel Electrophoresis and documentation

The PCR products were run on 1.5% agarose along with 100bp DNA molecular weight marker (New England Biolabs, USA) at 80V/cm and visualized using a gel documentation system (AlphaImager, Alpha Innotech, USA).

Results and Discussion

Prevalence

Out of 110 milk samples screened using SLS test, 108 (98.1%) milk samples showed positive reaction. Out of 108 SLS positive samples 85 yielded bacterial growth (78.7%). Out of these six (7.05%) were *E. faecalis* and four (4.70%) were *P. aeruginosa*. These organisms were identified on the basis of cultural characters, gram's staining, biochemical tests and MALDI-ToF. *E. faecalis* prevalence was found to be 7.05% which was in accordance with the study conducted by Ali et al. (2011) where they reported prevalence of *E. faecalis* from dairy buffaloes to be 3.17%. In another study Yang et al. (2019) observed *E. faecalis* prevalence of 4.5% from subclinical bovine mastitis and 6.36% from clinical mastitis (Awandkar et al. 2022). These findings were similar to the present study findings where a prevalence of less than 10% was observed. Since, *E. faecalis*, a major environmental mastitis-causing pathogen (Elhadidy and Elsayyad, 2013) has the ability to produce biofilm which causes inherent resistance for many antibiotics, thus its prevalence as well as antibiotic resistance profile needs to be examined on the regular basis.

P. aeruginosa prevalence was found to be 4.70 % which was in tandem with the findings of Sekhri et al. (2021) where they observed *P. aeruginosa* prevalence as 5.15%. Various studies too indicated prevalence of *P. aeruginosa* between 1-5% (Sharma and Sindhu, 2007, Banerjee et al, 2017, Yadav et al, 2020, Awandkar et al, 2022) similar to the findings of the present study.

Antibiotic sensitivity test

E. faecalis isolates showed sensitivity towards nalidixic acid (100%), streptomycin (83.33%), cepalothin, ciprofloxacin, co-trimaxazole, ceftriaxone, cefuroxime, gentamicin, oxacillin and vancomycin (66.66%) and resistance against penicillin G (100%), erythromycin, tetracycline (83.33%) and ampicillin/salbactam, ampicillin (66.66%). Similar results were observed by Frazzon et al. (2010) and Yang et al. (2019) where they reported that the isolates of *Enterococcus* spp. showed higher resistance against tetracycline (87.7%) and erythromycin (79.0%) which was similar to the present study findings as high resistance was observed against penicillin G (100%), erythromycin and tetracycline

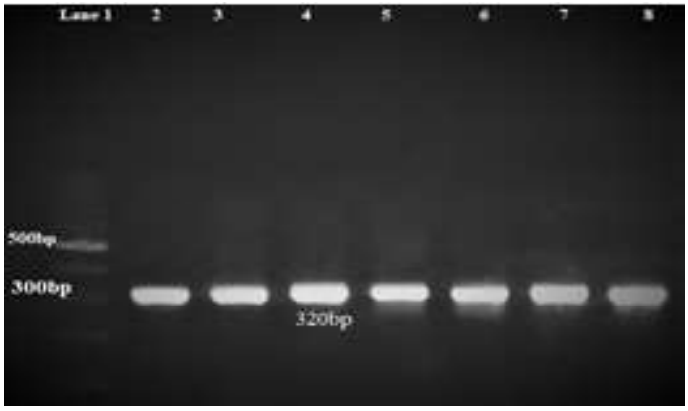

Fig. 1: Detection of *Enterococcus* spp. by PCR
Lane 1: 100bp DNA ladder; Lane 2-8: Positive samples

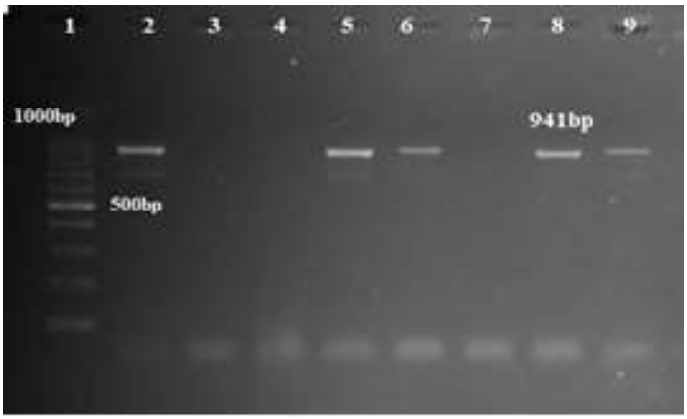

Fig. 2: Detection of *Enterococcus faecalis* by PCR
Lane 1: 100bp DNA ladder; Lane 2, 5, 6, 8 & 9 Positive; Lane 3-4 & 7 Negative

(83.33%). Similarly, Nam et al. (2010) observed that *Enterococcus* spp. isolates were sensitive to ampicillin, gentamicin and vancomycin, and resistant to ampicillin, tetracycline (69.5%), penicillin (64.7%), erythromycin (57.1%) and cephalothin (44.7%). Hamzah and Kadim (2018) results indicated resistance to vancomycin, penicillin, ofloxacin, ciprofloxacin, nitrofurantoin, tetracycline and amikacin whereas Gao et al. (2019) observed high resistance against penicillin, ceftiofur, tylosin, lincomycin, and oxytetracycline antibiotics in *Enterococcus* spp. which was similar to the findings of this study (Table 3).

P. aeruginosa isolates showed sensitivity towards cefuroxime, gentamicin, oxacillin (100%), amikacin, cefaperazone, erythromycin (25%) and resistance against ampicillin, ciprofloxacin, cephalothin, co-trimoxazole, ceftriaxone, nalidixic acid, penicillin G, streptomycin (100%), ampicillin/salbactam, amikacin, vancomycin, sparfloxacin, tetracycline, teicoplanin (75%) and amoxicillin, erythromycin (50%). *Enterococcus faecalis* isolates showed sensitivity towards nalidixic acid (100%), streptomycin (83.33%), cepalothin, ciprofloxacin, co-trimoxazole, ceftriaxone, cefuroxime, gentamicin, oxacillin and vancomycin (66.66%) and resistance against penicillin G (100%), erythromycin, tetracycline (83.33%) and ampicillin/salbactam, ampicillin (66.66%). The above results were similar to a study by Swetha et al. (2017) where they observed that isolates of *P. aeruginosa* from milk were resistant to ampicillin, penicillin, and oxacillin (100%) but sensitive to vancomycin (5.3%) and tetracycline (10.5%) indicating an alarming situation. Similarly Sekhri et al. (2021) studied antibiotic resistance of *P. aeruginosa* isolated from milk and observed that resistance to chloramphenicol, tetracycline, amoxicillin, erythromycin, cephalexin, teicoplanin (100%), azithromycin, doxycycline, ofloxacin, co-trimoxazole, vancomycin (80%), gatifloxacin, sparfloxacin (60%) and ciprofloxacin (50%), which was similar as findings of the present study (Table 3).

Polymerase chain reaction

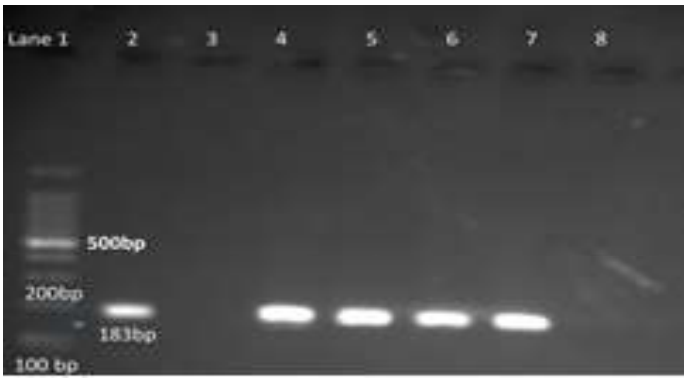

Fig. 3: Detection of *Pseudomonas aeruginosa* by PCR
Lane 1: Ladder; Lane 2, 4-7 Positive; Lane 3: Negative

On the basis of polymerase chain reaction using genus specific primers all the six isolates were identified as *Enterococcus* spp. producing a product size of 320bp (Fig.1). On the basis of species specific primer, all the isolates were *E. faecalis* producing a product size of 941bp (Fig. 2). Using genus specific primers all the six isolates were identified as *Enterococcus* spp. which was similar to El-Tawab et al. (2019), Devriese et al. (1996) and Jahan et al. (2013) where they used genus specific primers to confirm enterococci. Upon further analysis using species specific primers all the isolates were identified as *E. faecalis*. In various studies viz., Kariyama et al. (2000), Foka and Ateba (2019), Dutka-Malen (1995) and Mannu et al. (2003) used species specific primers for the confirmation of *E. faecalis* from mastitis milk. Similarly, Jahan et al. (2013) *E. faecalis* and *E. faecium* from meat and fermented meat products using species specific primer.

On the basis of polymerase chain reaction all the four isolates were *P. aeruginosa* producing a product size of 183bp (Fig. 3). In an earlier study Sekhri et al. (2021) used same species-specific primers for the confirmation of *P. aeruginosa* isolates. Similar to our study confirmation of *P.aeruginosa* using PCR has been

achieved by various earlier workers too (Iwasaki et al. 2019; Jangir et al. 2021; Schauer et al. 2021).

Detection of antibiotic resistance genes in *E. faecalis*

All the six *E. faecalis* isolates were tested for the presence of *vanA, vanB, vanC1, tetL, tetk* and *msrA/B* antibiotic resistant genes. It was revealed that one (16.66%) isolate was positive for *vanA* gene, three (50%) isolates were positive for *vanB* gene,

Table 1 Results of morphological, cultural and biochemical tests for *Enterococcus faecalis* identification

S. No.	Morphological & cultural characteristics	Interpretation
1	Growth on Bile Azide Esculin Agar	Small, round smooth dark brown colonies with black discoloration
2	Gram staining	Gram positive cocci (pairs or in chains)
3	Growth on Enterococcus Agar Base (1%TCC)	Maroon pin pointed coloured colonies
4	Growth on Blood Agar	Alpha or beta haemolytic
5	Sulphide Indole Medium Test	Non-Motile
	Biochemical Tests	Interpretation
1	Catalase	Negative
2	Oxidase	Negative
3	Indole	Negative
4	Methyl Red	Negative
5	Voges –Proskauer	Positive
6	Citrate Utilization	Negative
7	Urease	Negative
8	Nitrate reduction test	Positive
9	H_2S production	Negative
10	Esculin hydrolysis	Positive
11	Gas Production	Positive
12	6.5% NaCl (Salt tolerance)	Positive
13	Fermentation of sugar	
	a. Glucose	Positive
	b. Lactose	Positive
	c. Arabinose	Negative
	d. Sucrose	Positive
	e. Sorbitol	Positive
	f. Maltose	Positive

Table 2 Results of morphological, cultural and biochemical tests performed for *Pseudomonas aeruginosa* identification

S. No.	Morphological & cultural characteristics	Interpretation
1	Growth on BHI	Large round, opaque colonies with green discoloration.
2.	Growth on MLA	Pale colourless round colonies
3.	Gram's Staining	Gram Negative rods
4.	Growth on Cetrimide Agar	Cream medium sized flat, irregular edged colonies giving green pigmentation
5.	Sulphide Indole Medium Test	Motile
	Biochemical Tests	Interpretation
1	Catalase	Positive
2	Oxidase	Positive
3	Indole	Negative
4	Methyl Red	Negative
5	Voges-Proskauer	Negative
6	Citrate Utilization	Positive
7	Urease	Negative
8	Nitrate reduction test	Positive
9	ONPG	Negative

10	H_2S production	Negative
11	Arginine utilization	Positive
12	Triple sugar iron	K/K
13	Fermentation of sugar	
	a. Glucose	Positive
	b. Lactose	Negative
	c. Arabinose	Negative
	d. Sucrose	Negative
	e. Sorbitol	Negative
	f. Maltose	Negative

Table 3 Zone of antibiotic Resistance (Mean ± Standard Deviation) in mm in *Enterococcus faecalis* and *Pseudomonas aeruginosa* isolates

S. No.	Antibiotics	Mean ± SD in *Enterococcus faecalis* isolates (6)	Mean ± SD in *Pseudomonas aeruginosa* isolates (4)
1	Co-trimoxazole	17.2 ±8.5	5.3±6.1
2	Nalidixic Acid	11.2±10.2	7.0±9.5
3	Erythromycin	13.7±7.8	20.8±7.6
4	Tetracycline	14.5±2.4	14.8±2.5
5	Cephalothin	27.3±4.7	6.8±7.9
6	Streptomycin	10.2±5.1	19.3±7.0
7	Cefuroxime	24.0±4.7	4.3±8.5
8	Ceftriaxone	21.8±3.9	19.0±5.5
9	Teicoplanin	14.0±2.4	0.0±0.0
10	Enrofloxacin	23.8±4.7	30.0±3.6
11	Cefoperazone	20.7±6.6	17.5±4.2
12	Amikacin	16.3±1.2	18.0±8.1
13	Sparfloxacin	19.3±5.2	20.5±0.6
14	Ampicillin/sulbactam	28.2±3.5	0.0±0.0
15	Ciprofloxacin	19.8±4.0	30.8±1.5
16	Amoxicillin	0.0±0.0	0.0±0.0
17	Ampicillin	17.7±1.5	5.0±10.0
18	Oxacillin	14.8±7.5	0.0±0.0
19	Penicillin G	16.0±15.9	0.0±0.0
20	Vancomycin	15.2±1.2	0.0±0.0
21	Gentamicin	20.5±4.1	13.8±1.9

none of the isolates were positive for *vanCI* and *tetK*, five (83.3%) isolates were positive for *tetL* and all six (100%) isolates were positive for *msr A/B* gene. In an earlier study Erbas et al. (2016) observed one enterococci having *vanA* gene which was similar to the findings of the present study. Five (83.3%) isolates were positive for *tetL* was similar to the findings of a study by Jahan et al. (2013) where they stated that tetracycline efflux pumps *tetk* and *tetL* were positively amplified in 11 isolates. However, Huys et al. (2004) detected no *tetK* gene in their isolates, but Hummel et al. (2007) observed *tetL* in 94% and *tetK* in 56% isolates. In another study Stovcik et al. (2008) observed 63% isolates having *tetM* and 21% isolates having *tetL* genes whereas Frazzon (2010) observed 38% isolates having *tetM* and 9% having *tetL* and both *tetM* and *tetL* in 13% isolates, respectively. All the six (100%) isolates were positive for *msr A/B* gene which too is in tandem with the findings of Valenzuela, (2013) where they observed its presence in 66.66% isolates.

Upon comparison between phenotypic and genotypic resistance it was observed that for vancomycin phenotypically 66.66% isolates exhibited resistance while 50% isolates had *vanA*, *vanB* gene but none of the isolates had *vanC1* gene. For tetracycline, 83.33% isolates exhibited both phenotypic as well as genotypic resistance. For macrolide 83.33% isolates had phenotypic resistance while 100% isolates exhibited resistance genotypically.

Detection of antibiotic resistance genes in *P. aeruginosa*

All the four *P. aeruginosa* isolates were tested for the presence of *blaTEM, blaSHV, sulI, sulII, aadA, DHAM, MOXM, tetA, tetB, tetC, oxa-1, blaCTX-M, gyrA* and *gyrB* antibiotic resistant genes. It was revealed that three (75%) isolates were positive for *sulI*

Table 4 Primers used for the amplification of different organisms

S. No.	Organism	5' to 3'	Amplicon Size (bp)	Annealing Temp (°C)	Reference
1	*Enterococcus* rrs (16S rRNA)	F: GGATTAGATACCCTGGTAGTCC R: CGTTGCGGGACTTAACCCAAC	320	54	Devriese et al. (1996)
2	*E. faecalis*	F: ACGATTCAAAGCTAACTG R: ATCAAGTACAGTTAGTCT	941	54	Dutka-Malen et al. (1994)
3	*E. faecium*	F: TTGAGGCAGACCAGATTGACG R: TATGACAGCGACTCCGATTCC	658	54	Cheng et al. (1997)
4	*P. aeruginosa*	F: CTGGCCTTGACATGCTGAGA R:TCACCGGCAGTCTCCTTAGA	183	60	Sekhri et al. (2020)

Table 5 Sequence of primers used for the detection of antibiotic resistance genes in *Enterococcus faecalis*

S. No.	Antibiotics	Genes	Primers 5′-3′	Product (bp)	References
1.	Vancomycin	*vanA*	F:GCGAAAACGACAATTGC R:GTACAATGCGGCCGTTA	732	Dutka-Malen, (1995)
		vanB	F:ACGGAATGGGAAGCCGA R:TGCACCCGATTTCGTTC	647	Depardieu, (2004)
		vanC1	F:GGTATCAAGGAAACCTC R:CTTCCGCCATCATAGCT	822	Dutka-Malen, (1995)
2.	Tetracycline	*TetK*	F:TATTTTGGCTTTGTATTCTTTCAT R:GCTATACCTGTTCCCTCTGATAA	1159	Trzcinski et al. (2000)
		TetL	F:ATAAATTGTTTCGGTCGGTAAT R:AACCAGCCAACTAATGACAATGAT	1077	Trzcinski et al. (2000)
3	Macrolide	*MsrA/B*	F:R:GCAAATGCTGTAGGTAAGACAACT R:ATCATGTGATGTAAACAAAAT	400	Wondrack et al. (1996)

gene, one (25%) isolate was positive for *sulII* gene, two (50%) isolates were positive for *tetC* gene and all the 4 (100%) isolates were positive for *aadA*, *DHAM*, *gyrA* and *gyrB* gene. None of the isolates were positive for *blaTEM, blaSHV, blaCTX-M, oxa-1, tetA, MOXM and tetB* genes. All the four *Pseudomonas aeruginosa* isolates were tested for the presence of *blaTEM, blaSHV, sulI, sulII, aadA, DHAM, MOXM, tetA, tetB, tetC, oxa-1, blaCTX-M, gyrA* and *gyrB* antibiotic resistant genes.

It was revealed that three (75%) isolates were positive for *sulI* gene, one (25%) isolate was positive for *sulII* gene, two (50%) isolates were positive for *tetC* gene and all the 4 (100%) isolates were positive for *aadA*, *DHAM*, *gyrA* and *gyrB* gene. However, none of the isolates were positive for *blaTEM, blaSHV, blaCTX-M, oxa-1, tetA, MOXM and tetB* genes.

In a study Das et al. (2017) observed 6 (12%) isolates possessed *blaTEM* resistance genes in *P. aeruginosa* and but didn't find any isolate carrying *blaSHV* genes similar to present study. Similarly, Meng et al. (2020) reported four isolates having *sul1* gene among 44 isolates whereas in the present study three (75%) isolates were positive for *sulI* gene, one (25%) isolate was positive for *sulII* gene.

Upon comparison between phenotypic and genotypic resistance it was observed that for ampicillin/sulbactam, 75% isolates exhibited phenotypic resistance while 100% isolates exhibited genotypic resistance. For sulphonamides, aminoglycosides and fluroquinolones 100% isolates exhibited both phenotypic as well as genotypic resistance. For tetracycline, 75% isolates exhibited phenotypic resistance while 50% exhibited genotypic resistance.

Conclusions

From the present study, we can conclude that there is prevalence of environmental pathogens like *E. faecalis* 7.05% and *P. aeruginosa* 4.70% in mastitis. On the basis of antibiotic sensitivity test, these isolates showed multidrug resistance towards many commonly used antibiotics. Phenotypic and genotypic resistance when compared revealed that there is partial correlation between these.

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Table 6 Sequence of primers used for the detection of antibiotic resistance genes in *Pseudomonas aeruginosa*

S No.	Antibiotics	Genes	Primers 5′-3′	References
1.	Beta lactam	*BlaTEM*	F: GAGTATTCAACATTTTCGT R: ACCAATGCTTAATCAGTGA	Maynard et al. (2004)
		blaSHV	F:TCGCCTGTGTATTATCTCCC R:CGCAGATAAATCACCACAATG	
		blaCTX-M	F:TTTGCGATGTGCAGTACCAGTAA R:CGATATCGTTGGTGGTGCCATA	Edelstein (2003)
		OXA-1	F:ACACAATACATATCAACTTCGC R:AGTGTGTGTTTAGAATGGTGATC	Oliver et al. (2002)
2.	Tetracycline	*TetA*	F:CGATCTTCCAAGCGTTTGTT R:CCAGAAGAA CGAAGCCAGTC	Faldynova et al. (2013)
		TetB	F:TACAGGGATTATTGGTGAGC R:ACATGAAGGTCATCGATAGC	
		TetC	F:ACTTGGAGCCACTATCGAC R:CTACAATCCATGCCAACCC	Maynard et al. (2004)
3.	Sulphonamide	*SulI*	F:TTCGGCATTCTGAATCTCAC R:ATGATCTAACCCTCGGTCTC	Shehata et al. (2016)
		SulII	F:CGGCATCGTCAAACATAACC R:GTGTGCGGATGAAGTCAG	Maynard et al. (2004)
4.	Ampicillin	*MOXM*	F:GCTGCTCAAGGAGCACAGGAT R:CACATTGACATAGGTGTGGTGC	Van et al. (2008)
		DHAM	F:AACTTTCACAGGTGTGCTGGGT R:CCGTACGCATACTGGCTTTGC	
5.	Streptomycin	*aadA*	F:TGATTTGCTGGTTACGGTGAC R:CGCTATGTTCTCTTGCTTTTG	
6.	Fluoroquinolone	*gyrA*	F:GGATAGCGGTTAGATGAGC R:CGTTAATCACTTCCGTCAG	Yue et al. (2008)
		gyrB	F:CAGCAGATGAACGAACTGCT R: AACCAAGTGCGGTGATAAGC	

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