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Short Paper

Antibiotic sensitivity patterns in *Staphylococcus* spp. isolated from goat milk in association with molecular detection of antibiotic resistance genes

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Abstract

Background: Microbes become unresponsive to the drugs used to treat infections due to their ability to acquire antimicrobial resistance. **Aims:** The present research aimed to study the patterns of phenotypic and genotypic antimicrobial resistance in *Staphylococcus* spp. isolated from goat milk. **Methods:** A total of 200 milk samples were collected to isolate *Staphylococcus* spp. from mastitic and healthy goats from Punjab state, India. The isolates were then identified biochemically and molecularly (polymerase chain reaction (PCR)). An antibiotic sensitivity test was conducted using 15 different antibiotics. Molecular detection of antibiotic resistance genes was done using PCR. Chi-square test was done to study the association between genotypic and phenotypic resistance patterns among the isolates. **Results:** A total of 47 *Staphylococcus* spp. were isolated of which 33 and 14 isolates were respectively coagulase positive and negative. The isolates phenotypically showed the highest resistance to Penicillin G (P) (91.4%). Methicillin resistant *Staphylococcus aureus* (MRSA) was found 56.25%. Amongst the antibiotic resistance genes, *tet*K (87.23%) was the most prevalent isolated gene followed by *bla*Z (85.10%), *mec*A (48.93%), and *tet*M (14.89%). Statistical analysis revealed that the genotypic and phenotypic resistance patterns were significantly associated with penicillin and methicillin (MET) resistances. **Conclusion:** The high prevalence of antibiotic-resistant *Staphylococcus* spp., especially MRSA, in goat milk is of concern and needs to be addressed in this area.

Key words: Antibiotic resistance, Antibiotic resistance genes, Goat, PCR, *Staphylococcus* spp.

Introduction

Antibiotics are the substances used to kill or stop the growth of microbes and can be considered miraculous drugs in treatment of infectious diseases, but the increasing use of antibiotics in humans and animals has led to the development of antimicrobial resistance (WHO, 2001).

Raw goat's milk can be considered as a major source of antibiotic resistant pathogens from animal, human or environmental origin. *Staphylococcus aureus* is the main cause of clinical mastitis in small ruminants among which dairy goat farms burden severe economic losses due to staphylococcal intramammary infections (Bergonier *et al*., 2003).

The intramammary antibiotics to treat subclinical mastitis (SCM) of dry small ruminants has increased in the farms (Poutrel *et al*., 1997; Santis *et al*., 2001), leading to emerging resistant strains, incrementally (Walther *et al*., 2006). To choose effective antibiotics and to identify the resistance patterns of multi-drug resistance strains in farms, evaluation of antimicrobial susceptibility in *Staphylococcus* spp. isolated from goat milk is important (Virdis *et al*., 2010).

Antibiotic resistance threatens animals and public health. Milk and its products containing multi-resistant bacteria can act as a potential source of the resistant strains which can transmit to humans (Gundogan *et al*., 2006). Goat milk is a valuable nutritious food highly demanded from people. Some studies of antimicrobial resistance have been conducted in Punjab, India, however, little data is available from goats. As antibiotic resistance patterns change over time, this study will inform us of the current status of antibiotics resistance in goats' milk to consider better therapeutic approaches.

Materials and Methods

Sampling

A total of 200 milk samples were collected aseptically from goats herds (healthy and mastitic), located in the Punjab state of India. Sampling was from rural areas of different districts of the state, including Ludhiana district (n=90), Kapurthala (n=50), and

Sangrur (n=60) from where 10 animals were screened from each of the 20 selected herds. These samples were then transferred to the Laboratory of Veterinary Microbiology, GADVASU in icebox for further processing.

Processing of milk samples

Sodium lauryl sulphate (SLS) test was carried out on the samples to confirm the mastitic status of the animal (Kour *et al*., 2017). The samples were then inoculated on various media consisting of Brain heart infusion agar, Blood agar, Mannitol salt agar, and Baird Parker agar for isolation of the organism. All the media were acquired from Himedia labs, Mumbai, India. The suspected colonies on different media were subjected to Gram staining for detection of the isolates.

Biochemical identification

Isolates were identified by different biochemical tests including catalase, oxidase, and slide coagulase. Species identification was done using HiStaph identification kit (Himedia, Mumbai, India) comprising different biochemical tests: Voges Proskauer, Alkaline phosphatase, ortho-Nitrophenyl-β-galactoside (ONPG), Urease, Arginine utilization and utilization of different sugars viz. Mannitol, Sucrose, Lactose, Arabinose, Raffinose, Trehalose, and Maltose.

Antibiogram study

The isolates were subjected to antibiotic sensitivity testing using Kirby Bauer disc diffusion method. Fifteen different antibiotics tested included chloramphenicol (C) (30 mcg), penicillin G (P) (10 units), tetracycline (TE) (30 mcg), amoxyclav (AMC) (30 mcg), cotrimoxazole (COT) (25 mcg), gentamicin (GEN) (10 mcg), erythromycin (E) (15 mcg), methicillin (MET) (5 mcg), enrofloxacin (EX) (10 mcg), cephalothin (CEP) (30 mcg), doxycycline (DO) (30 mcg), ciprofloxacin (CIP) (5 mcg), azithromycin (AZM) (15 mcg), gatifloxacin (GAT) (5 mcg), and streptomycin (S) (10 mcg). The isolates were designated as resistant, intermediate or susceptible to antibiotics as per Clinical and Laboratory Standards Institute (CLSI) guidelines.

DNA extraction by hot-cold lysis method

Pure culture colonies (4-5) were taken into 400 µL of sterile distilled water/normal saline in a microtube placed into a water-bath (preheated to 100°C) for 10 min and then immediately transferred to ice kept at -20°C. The suspension was centrifuged and the supernatant was used as a source of DNA (Arora *et al*., 2006).

Genus specific polymerase chain reaction (PCR) for detection of *Staphylococcus* spp.

Polymerase chain reaction was carried out to target *16S rRNA* gene using genus specific primers (F: CTG TAC GCT AGG TGG AGC G; R: TTT TGC AGG ATG TCC GCC TT) (Kour *et al*., 2017). An amplification reaction mixture (25 µL) was prepared using PCR mastermix (Promega) (12.5 µL), 1 µL each of forward and reverse primer (20 pM/µL), template DNA (5 µL) and nuclease free water (NFW) (5.5 µL). Polymerase chain reaction cycling conditions included initial denaturation (94°C, 5 min) and 30 cycles of denaturation (94°C, 45 s), annealing (55°C, 45 s), extension (72°C, 1 min), and final extension (72°C, 10 min).

Detection of antibiotic resistance genes for *Staphylococcus* spp.

All the *Staphylococcus* isolates were tested for the presence of *blaZ*, *tetM*, *tetK*, and *mecA* antibiotic resistance genes encoding β-lactam resistance, TE resistant protein, TE efflux pump, and MET resistance, respectively. Amplification of the desired genes was accomplished using specific primers for each gene (Table 1). An amplification reaction mixture (25 µL) was prepared using PCR mastermix (Promega) (12.5 µL), 1 µL each of forward and reverse primer (20 pM/µL), template DNA (5 µL), and NFW (5.5 µL). Polymerase chain reaction cycling conditions comprised of initial denaturation (94°C, 5 min) followed by 30 cycles (35 cycles for *tet*K), each of denaturation (94°C, 45 s and 30 s for *tet*K), annealing (55°C, 45 s and 30 s for *tet*K), extension (72°C, 1 min and 30 s in case of *tet*K), and final extension (72°C, 7 min and 4 min for *tet*K).

Analysis of PCR product

The amplified products were subjected to electrophoresis at 79 V for 1 h using 1.5% agarose gel and were finally visualised using gel documentation system.

Statistical analysis

Chi-square test was used to determine the correlation between phenotypic and genotypic resistance of the

Table 1: Sequence of primers used to detect antibiotic resistance genes

Ser. No.	Antibiotic resistance gene	Oligonucleotide sequence (5´-3´)	Size (bp)	Reference
1	Methicillin (*mec*A)	F: CCTAGTAAAGCTCCGGAA R: CTAGTCCATTCGGTCCA	314	Choi *et al*. (2003)
2	β-lactam (*bla*Z)	F: ACTTCAACACCTGCTGCTTTC R: TGACCACTTTTATCAGCAACC	173	Martineau *et al*. (2000)
3	Tetracycline (*tet*M)	F: AGTGGAGCGATTACAGAA R: CATATGTCCTGGCGTGTCTA	158	Strommenger *et al*. (2003)
4	Tetracycline (*tet*K)	F: GTAGCGACAATAGGTAATAGT R: GTAGTGACAATAAACCTCCTA	360	Strommenger *et al*. (2003)

organism to various antimicrobial agents. The tests were considered significant at 1% level of significance (P<0.01) and a 5% level of significance (P<0.05).

Results

Detection of *Staphylococcus* spp.

Of 200 milk samples processed, 47 isolates of *Staphylococcus* spp. were obtained and identified biochemically. Of the 47 isolates, 33 (70.2%) were coagulase positive *Staphylococcus* spp. which comprised of *S. aureus* (n=16; 48.48%), *S. hyicus* (n=14; 42.42%), and *S. intermedius* (n=3; 9.09%). Out of the total isolates, 14 (29.78%) were coagulase negative comprising of *S. epidermidis* (n=4; 28.57%), *S. hominis* (n=2; 14.28%), *S. felis* (n=2; 14.28%), *S. saprophyticus* (n=2; 14.28%), *S. capitis* subsp. *capitis* (n=1; 7.14%), *S. carnosus* subsp. *utilis* (n=1; 7.14%), *S. hemolyticus* (n=1; 7.14%), and *S. caseolyticus* (n=1; 7.14%). In PCR, an amplicon size of 532 bp was obtained in the positive control (*S. aureus* ATCC 12600) and isolates (Fig. 1).

Antibiogram study

Overall antibiotic sensitivity pattern for *Staphylococcus* spp. is given in Table 2. Among the coagulase negative *Staphylococcus* spp., the highest resistance belonged to P (92.85%) followed by MET (64.28%) and COT (50%).

Staphylococcus aureus and *S. hyicus* isolates showed the highest resistance to P (100% and 78.57%, respectively) followed by MET (56.25% and 71.24%, respectively).

Among *S. intermedius* isolates, the highest resistance was found to P (100%) followed by 33.3% resistance to MET, GEN, EX, AMC, CIP, COT, GAT, and S.

PCR amplification of antibiotic resistance genes

Of the 47 isolates, 23 isolates (48.93%) were positive for *mec*A gene, 40 isolates (85.10%) for *bla*Z, 7 isolates (14.89%) for *tet*M and 41 isolates (87.23%) were positive for the presence of *tet*K gene (Fig. 2).

An amplicon size of *mec*A gene was 314 bp. The isolates were found *mec*A positive included *S. aureus* (n=7; 43.75%), *S. hyicus* (n=6; 42.85%), *S. intermedius* (n=3; 100%), and coagulase negative staphylococci (CoNS) (n=7; 50%). Among coagulase negative species,

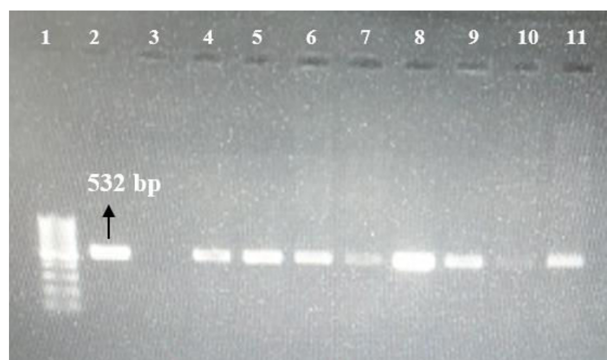

Fig. 1: Gel electrophoresis of PCR amplifying fragments of *Staphylococcus* spp. using specific primer pair. Lane 1: 100 bp DNA ladder, Lane 2: Positive control (*S. aureus* ATCC 12600), Lane 3: Negative control, and Lane 4-11: Positive samples

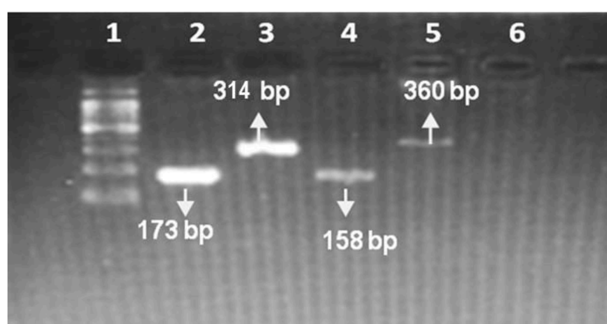

Fig. 2: PCR amplification of antibiotic resistance genes (*bla*Z, *mec*A, *tet*M, and *tet*K) for *Staphylococcus* spp. Lane 1: 100 bp DNA ladder, Lane 2: *bla*Z gene, Lane 3: *mec*A gene, Lane 4: *tet*M gene, Lane5: *tet*K gene, and Lane 6: Negative control

Table 2: CST pattern of *Staphylococcus* spp.

Antibiotic	S (No.)	S (%)	R (No.)	R (%)	I (No.)	I (%)
Methicillin (MET)	7	14.8	29	61.7	11	23.4
Penicillin G (P)	4	8.5	43	91.4	-	0
Gentamicin (GEN)	39	82.9	3	6.38	5	10.63
Enrofloxacin (EX)	40	85.1	7	14.89	-	0
Erythromycin (E)	22	46.8	4	8.5	21	44.6
Tetracycline (TE)	44	93.6	2	4.25	1	2.12
Amoxyclav (AMC)	38	80.8	9	19.1	-	0
Chloramphenicol (C)	41	87.2	-	0	6	12.7
Doxycycline (DO)	43	91.4	4	8.5	-	0
Ciprofloxacin (CIP)	43	91.4	2	4.25	2	4.25
Cotrimoxazole (COT)	38	80.8	8	17.02	1	2.12
Gatifloxacin (GAT)	31	65.9	5	10.63	11	23.4
Azithromycin (AZM)	39	82.9	2	4.25	6	12.7
Streptomycin (S)	41	87.2	6	12.7	-	0
Cephalothin (CEP)	41	87.2	1	2.12	5	10.63

S (No.): No. of isolates sensitive to particular antibiotic, S (%): Percentage of isolates showing sensitivity to particular antibiotic, R (No.): No. of isolates resistant to particular antibiotic, R (%): Percentage of isolates showing resistance to particular antibiotic, I (No.): No. of isolates showing intermediate sensitivity to particular antibiotic, and I (%): Percentage of isolates showing intermediate sensitivity to particular antibiotic. CST: Culture sensitivity test

Table 3: Number of species isolates showing both phenotypic and genotypic resistance to the antibiotics

Species	Methicillin resistant and *mec*A positive	Penicillin resistant and *bla*Z positive	Tetracycline resistant and *tet*M positive	Tetracycline resistant and *tet*K positive
S. aureus	7	12	0	2
S. hyicus	6	11	0	0
S. intermedius	3	2	0	0
S. epidermidis	1	2	1	1
S. hominis	2	2	0	0
S. saprophyticus	2	2	0	0
S. hemolyticus	1	1	0	0
S. caseolyticus	0	1	0	0
S. carnosus subsp. *utilis*	0	1	0	0
S. capitis subsp. *capitis*	1	1	0	0
S. felis	0	2	0	0

S. epidermidis (n=1; 25%), *S. hominis* (n=2; 100%), *S. saprophyticus* (n=2; 100%), *S. hemolyticus* (n=1; 100%), and *S. capitis* subsp. *capitis* (n=1; 100%), were positive for *mec*A.

On amplification of *bla*Z gene, an amplicon size of 173 bp was obtained. *Staphylococcus aureus* (n=14; 87.5%), *S. hyicus* (n=12; 85.7%), *S. intermedius* (n=2; 66.6%), and CoNS (n=12; 85.7%) were positive for *bla*Z gene. Among coagulase negative species, *S. epidermidis* (n=2; 50%), *S. hominis* (n=2; 100%), *S. saprophyticus* (n=2; 100%), *S. hemolyticus* (n=1; 100%), *S. capitis* subsp. *capitis* (n=1; 100%), *S. felis* (n=2; 100%), *S. carnosus* subsp. *utilis* (n=1; 100%), and *S. caseolyticus* (n=1; 100%) were positive for *bla*Z.

On amplification of *tet*M gene in PCR, an amplicon size of 158 bp was obtained. *Staphylococcus aureus* (n=2; 12.5%), *S. hyicus* (n=4; 28.57%), and CoNS (n=1; 7.14%) were positive for *tet*M gene. Among coagulase negative species, *S. epidermidis* (n=1; 25%), were positive for *tet*M.

On amplification of *tet*K gene in PCR, an amplicon size of 360 bp was obtained. *Staphylococcus aureus* (n=15; 93.75%), *S. hyicus* (n=12; 85.71%), *S. intermedius* (n=3; 100%), and CoNS (n=11; 78.57%) were positive for *tet*K. Among coagulase negative species, *S. epidermidis* (n=3; 75%), *S. hominis* (n=2; 100%), *S. saprophyticus* (n=2; 100%), *S. hemolyticus* (n=1; 100%), *S. capitis* subsp. *capitis* (n=1; 100%), *S. carnosus* subsp. *utilis* (n=1; 100%), and *S. caseolyticus* (n=1; 100%) were positive for *tet*K.

The number of species showing both phenotypic and genotypic resistance to the tested antibiotics are given in Table 3.

Statistical analysis

Using χ^2 test, highly significant association was found between genotypic and phenotypic resistance to penicillin and MET at 1% level of significance.

Discussion

Antibiotic resistance threatens both animals and humans. Antibiotic resistance patterns keep on changing from time to time, so, antibiogram assessments of the organisms are important. Resistance studies on *Staphylococcus* spp. have been carried out by researchers, previously. The results of the disk diffusion method according to CLSI guideline showed that the resistance of the isolated *Staphylococcus* spp. to P, TE, E, GEN, and EX was 26.9%, 7.5%, 6.0%, 3.0%, and 1.5%, respectively (Ünal *et al*., 2012). Species of *S. epidermidis* (87.5%), *S. caprae* (6.2%), *S. aureus* (4.2%), and *S. simulans* (2.1%) were isolated from milk of healthy goats by Ruiz *et al*. (2016) among which the highest level of antibiotic resistance was observed in *S. epidermidis* and *S. aureus*. In our study, the highest resistance to P was observed, which can be attributed to excessive and long-term use of the drug to treat mastitis (Sree and Ayodha, 2016). Also, the present study revealed that the new generation drugs like levofloxacin, EX, C, and GEN were effective in the treatment of staphylococcal mastitis, which was in accordance with studies conducted by (Sarangi *et al*., 2009). In Rola *et al*. (2015) study, 6.3% of coagulase positive *Staphylococcus* spp. isolated from raw goats' milk showed resistance to TE and cefoxitin. Moroni *et al*. (2004) observed that all β-lactams (except cefoperazone) were effective against *S. epidermidis* and *S. caprae*, whereas other antibiotics were either less effective or showed no activity.

Rahmdel *et al*. (2018) conducted PCR on staphylococci isolated from sheep and goats' milk and found the occurrence of antibiotic resistance genes in order of *blaZ*/*tet*K (100%), *mec*A/*erm*B (86%), *erm*C (50%), and *tet*M (18%).

Georgopapadakou (1993) reported that the presence of altered penicillin binding protein or excessive production of β-lactamase, can be attributed to the presence of isolates exhibiting phenotypically positive to β-lactam resistance but genotypically negative to *mec*A.

According to Franca *et al*. (2012), the main mechanism of β-lactams resistance in staphylococci causing mastitis in goats and sheep might be due to the production of β-lactamase encoded by *bla*Z gene. The phenotypic resistance to penicillin in absence of *blaZ* gene could be due to the fact that *Staphylococcus* spp. can develop tolerance to the β-lactam antibiotics (Sabath, 1979; Sabath, 1982) or due to reduced affinity of some additional protein to the β-lactam antibiotics (Brown and Reynold, 1980).

In the present study, it was found that some isolates

harboring the TE resistance genes were phenotypically sensitive to the drug. This can be due to the less expression of *tet*M and *tet*K genes (Martineau *et al*., 2000; Choi *et al*., 2003; Huys *et al*., 2005; Emaneini *et al*., 2013).

Furthermore, the divergence between genotypic and phenotypic resistances can be attributed to the fact that all possible resistance genes were not screened, and also some genes responsible for resistance may not be activated in certain isolates.

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Conflict of interest

The authors declare that they have no competing interests.

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