PLASMID PROFILE AND ANTIMICROBIAL RESISTANCE PATTERN OF COAGULASE NEGATIVE STAPHYLOCOCCI (CNS) BACTERIA ISOLATED FROM BOVINE SUBCLINICAL MASTITIS

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ABSTRACT

Coagulase-negative staphylococci (CNS), a heterogeneous conglomeration of several species of staphylococcus bacteria, are the predominant cause of bovine subclinical mastitis (SCM). The species diversification of CNS involved in bovine subclinical mastitis, their relationship with plasmid configuration and antibiotic resistance, has been portrayed in this paper, to supplement the existing scanty literature on the subject. This study, undertaken on the plasmid profile of 125 CNS microorganisms, isolated from cows with subclinical mastitis (SCM) from seven dairy farms in Maharashtra, India, revealed that 98 strains of CNS (78.4%) contained plasmids. Majority of the strains (90.8%) contained single plasmid (26083 bp). The number of plasmids was 2 (24500 bp and 1135 bp) and 3 (22826 bp, 1135 bp, and 741 bp) in 3.06% and 6.1% of the strains respectively. Ten antibiotics were used in this study to assess the antibiotic resistance pattern of the isolates. There was difference in the antibiotic resistance pattern of CNS microorganisms recovered from different farms, so also the number of plasmids carried by them. Six species of CNS, *viz*., *S. xylosus* (43.20%), *S. epidermidis* (29.60%), *S. chromogenes* (11.20%), *S. simulans* (8.00%), and *S. lentus* and *S. capitus* (4% each) were isolated. All the species of CNS showed multi-drug resistance, but it was different in different species. The strains were highly sensitive (96.8%) to chloramphenicol, and highly resistant (99.2%) to penicillin. The difference in antibiotic resistance pattern in different farms, in strains bearing different number of plasmids, and in different species of CNS, implied its epidemiological significance. The similarity in antibiotic resistance pattern of structurally different plasmids suggested inter-species exchange of antibiotic resistance genes, along with plasmids.

KEY WORDS

Antibiogram, Bovine, CNS, Plasmid, Subclinical mastitis

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INTRODUCTION

Bovine subclinical mastitis (SCM) is a leading cause of economic despondency as a result of fall in milk production and curtailment of the productive life of cow. Coagulase-negative staphylococci (CNS), a heterogeneous conglomeration of several species of staphylococcus bacteria, are the predominant cause of SCM (Krithiga *et al*., 2011).

Bacteria contain plasmids, which are covalently linked to closed extra chromosomal DNA material inside the bacterial cell. Plasmids carry antimicrobial resistance encoding genes (Rosander *et al*., 2008). Increase in the number of plasmids in *Escherichia coli*, isolated from poultry meat was found to be linked with multi-drug resistance (Londhe *et al*., 2013). However, there have been scant studies on the relationship between plasmid configuration and antibiotic resistance in different species of CNS, so also the species difference in antibiotic resistance pattern (Waller *et al*., 2011).

The purpose of this study was to investigate the species of CNS bacteria involved in SCM in cows, their plasmid profile and molecular characterization, and antibiotic resistance pattern.

MATERIALS AND METHODS

A total of 125 coagulate-negative staphylococci (CNS), isolated from cows with subclinical mastitis (SCM) from seven dairy farms in Maharashtra, and maintained in glycerol broth, in the Department of Veterinary Microbiology, Nagpur Veterinary College, Nagpur, India, were used in the present investigation. The isolates were identified on the basis of cultural, morphological, and biological characters as per the procedure of Cowan and Steel (1970) and Cruickshank *et al*. (1975).

The plasmids were isolated by alkaline lysis method by using lysozyme (Chousalkar, 2004). The genomic DNA of the plasmids was isolated, and their profile was studied by agarose gel electrophoresis as per Sambrook and Russel (2001). The image of plasmid DNA was pictured under UV illuminator with gel documentation system (Vilber Lourmat, France).

Commercially available antibiotics discs (Himedia laboratories Ltd., Mumbai) were used for determining *in vitro* antibiotic susceptibility of individual isolates by single disc diffusion method (Bauer *et al.,* 1966). Ten antibiotics *viz*., Ampicillin (Am), Chloramphenicol (Cl), Ciprofloxacin (Cp), Cloxacillin (Cx), Gentamycin (Gm), Methicillin (Mc), Novobiocin (Nb), Penicillin (Pc), Streptomycin (Sm), and Tetracycline (Tc) were used at different concentrations per disc. The concentrations of antibiotics per disc used in the study were: Am (10 mcg), Cl (30 mcg), Cp (10 mcg), Cx (10 mcg), Gm (10 mcg), Mc (30 mcg), Nb (30 mcg), Pc (10 IU), Sm (25 mcg), and Tc (30 mcg).

The nutrient broth was inoculated with single isolated colony, and the tubes were incubated at 37°C for 16–18 hours. The broth cultures were uniformly spread over Muller-Hington agar plates using sterile cotton swabs. The inoculum was allowed to dry, and the antibiotic discs were placed on

it using sterile forceps. The plates were then incubated and observed after overnight incubation. The diameter of zones of inhibition was measured to the nearest millimeter and interpretation was made as per the zone size interpretation chart provided by the manufacturer.

RESULTS

Out of 125 strains of CNS isolated from various farms, 98 strains (78.4%) harboured plasmids (Table-1). Most of the strains (90.8%) contained single plasmid. The number of plasmids was 2 and 3 in 3.06% and 6.1% of the strains respectively.

The antibiotic resistance pattern of CNS microorganisms in different farms was different. Farm A cows were resistant to eight antibiotics (Am, Cf, Cx, Gm, Mc, Pc, Sm, Tc), and sensitive to two antibiotics (Cl, Nb**)**. Farm B cows were resistant to eight antibiotics (Am, Cx, Gm, Mc, Nb, Pc, Sm, Tc), and sensitive to two antibiotics (Cl, Cp). Farm C cows were resistant to seven antibiotics (Am, Cx, Gm, Mc, Pc, Sm, Tc), and sensitive to three antibiotics (Cl, Cp, Nb). Farm D cows were resistant to six antibiotics (Am, Gm, Nb, Pc, Sm, Tc), and sensitive to four antibiotics (Cl, Cp, Cx, Mc). Farm E cows were resistant to six antibiotics (Am, Cx, Gm, Mc, Pc, Sm), and sensitive to four antibiotics (Cl, Cp, Cx, Mc). Farm F cows were resistant to six antibiotics (Am, Cx, Mc, Nb, Pc, Sm), and sensitive to four antibiotics (Cl, Cp, Gm, Tc). Farm G cows were resistant to six antibiotics (Am, Cx, Gm, Mc, Pc, Sm), and sensitive to four antibiotics (Cl, Cp, Nb, Tc).

The molecular characteristic of the plasmids are depicted in Plate-1. Eighty nine (89) strains recovered from farms A, B, C, D, E, F, and G contained single plasmid of 26083 bp. Three (3) strains from farms A and G contained two plasmids of 24500 bp and 11351 bp and 6 strains from farms A, B, E, and F contained three plasmids of 22826 bp, 1135 bp, and 741 bp.

The antibiotic resistance pattern of CNS strains (Table-2) revealed that the strains having single plasmid of 26083 bp were resistant to ampicillin, cloxacillin, gentamicin, methicillin, penicillin, streptomycin, and tetracycline. The strains bearing 2 plasmids of 24500 bp and 1135 bp were resistant to ampicillin, gentamicin, methicillin, penicillin, and streptomycin. The strains bearing 3 plasmids of 22826 bp, 1135 bp, and 741 bp were resistant to ampicillin, gentamicin, methicillin, penicillin, and streptomycin.

Six species of CNS microorganisms *viz*., *S. xylosus, S. epidermidis, S. chromogenes, S. simulans, S. lentus, and S. capitis* were isolated from 125 samples constituting 43.2, 29.6, 11.2, 8.0, 4.0, and 4.0 percents respectively (Figure-1). The incidence of SCM due to *S. xylosus* was the highest (43.2%) followed by *S. epidermidis* (29.6%).

The antibiotic resistance pattern (Table-3) revealed that *S. xylosus* showed resistance to all the ten antibiotics used. *S. epidermidis* was resistant to 9 out of 10 antibiotics used. It did not exhibit resistance against novobiocin (0%). *S. chromogenes* was resistant to 8 out of 10 antibiotics used. It did not exhibit resistance against chloramphenicol (0%) and novobiocin (0%). *S. simulans* was resistant to five antibiotics. It did not exhibit resistance against Cl, Cx,

Gm, Mc, and Nb (0% each). *S. lentus* was resistant to 9 out of 10 antibiotics used. It did not exhibit resistance against Cl (0%). *S. capitis* was resistant to 6 out of 10 antibiotics used. It did not exhibit resistance to Cl, Cx, Cp, and Nb (0% each).

Table-1. Plasmid profile of CNS strains in different farms.

Farm	No. of CNS strains	No. of plasmid bearing strains	No. of Plasmids One	Two	Three	Antibiotic Resistant Pattern
A	12	9	6	1	2	Am, Cp, Cx, Gm, Mc, Pc, Sm, Tc
B	35	30	29	0	1	Am, Cx, Gm, Mc, Nb, Pc, Sm, Tc
C	10	9	9	0	0	Am, Cx, Gm, Mc, Pc, Sm, Tc
D	6	3	3	0	0	Am, Gm, Nb, Pc, Sm, Tc
E	25	17	15	0	2	Am, Cx, Gm, Mc, Pc, Sm
F	8	7	6	0	1	Am, Cx, Mc, Nb, Pc, Sm
G	29	23	21	2	0	Am, Cx, Gm, Mc, Pc, Sm
Total	125	98	89	3	6	---

Abbreviation of antibiotics: Ampicillin (Am), Chloramphenicol (Cl), Ciprofloxacin (Cp), Cloxacillin (Cx), Gentamycin (Gm), Methicillin (Mc), Novobiocin (Nb), Penicillin (Pc), Streptomycin (Sm), Tetracycline (Tc).

Table-2. Antibiotic resistance pattern of CNS strains with different number of plasmids.

No. of Plasmids	No. of Strains	Molecular weight (bp)	Farms	Antibiotic resistance pattern
1	89	26083	A, B, C, D, E, F, G	Am, Cx, Gm, Mc, Pc, Sm, Tc
2	3	24500, 1135	A,G	Am, Gm, Mc, Pc, Sm
3	6	22826, 1135, 741	A, B, E, F	Am, Gm, Mc, Pc, Sm

Abbreviation of antibiotics: As in Table-1.

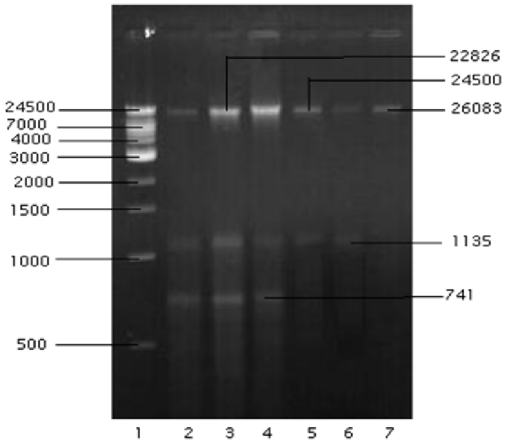

Plate-1. Plasmid profile of different strains of CNS.

Lane 1: Supermix DNA ladder (500 bp), Lanes 2-4: 3 plasmids (22826, 1135 & 741 bp) of CNS, Lanes 5-6: 2 plasmids (24500 & 1135 bp) of CNS, Lane 7: 1 plasmid (26083 bp) of CNS

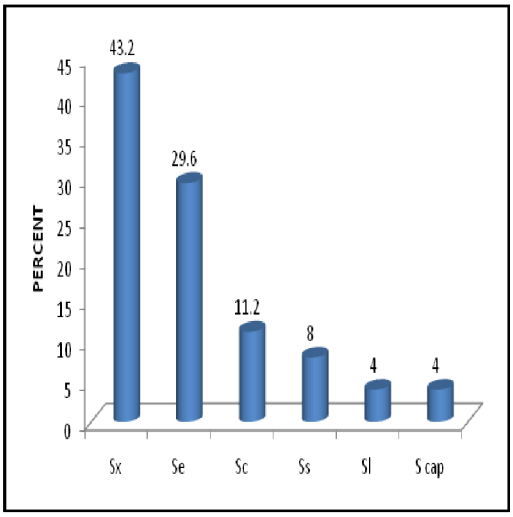

Figure-1. Distribution of different species of CNS.

Table-3. Antibiotic resistance (i*n vitro*) of CNS strains isolated from subclinical mastitis.

Anti-biotic	Species of Coagulase-negative Staphylococci						Total
	S. xylosus (N=54)	*S. epidermidis* (N=37)	*S. chromogenes* (N=14)	*S. simulans* (N=10)	*S. lentus* (N=5)	*S. capitis* (N=5)	(N=125)
Am	53 (98.14)	37 (100)	13 (92.85)	10 (100)	5 (100)	5 (100)	123 (98.40)
Cl	3 (5.55)	1 (2.70)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	4 (3.20)
Cx	32 (59.25)	17 (45.94)	6 (42.85)	0 (0.00)	4 (80.00)	0 (0.00)	59 (47.20)
Cp	17 (31.48)	9 (24.32)	3 (21.42)	3 (30.00)	1 (20.00)	0 (0.00)	33 (26.40)
Gm	35 (64.81)	21 (56.75)	4 (28.57)	0 (0.00)	1 (20.00)	1 (20.00)	62 (49.60)
Mc	39 (72.22)	27 (72.97)	7 (50.00)	0 (0.00)	4 (80.00)	1 (20.00)	78 (62.40)
Nb	54 (100)	0 (0.00)	0 (0.00)	0 (0.00)	4 (80.00)	0 (0.00)	58 (46.40)
Pc	53 (98.14)	37 (100)	14 (100)	10 (100)	5 (100)	5 (100)	124 (99.20)
Sm	44 (81.48)	23 (62.16)	7 (50.00)	5 (50.00)	3 (60.00)	3 (60.00)	85 (68.00)
Tc	20 (37.03)	8 (21.62)	8 (57.14)	4 (40.00)	3 (60.00)	4 (80.00)	47 (37.60)

Note: (1) The figures in parentheses are percentages. (2) The abbreviations of antibiotics are same as in Table-1. (3) The concentration of antibiotics per disc used in the study were: Am (10 mcg), Cl (30 mcg), Cp (10 mcg), Cx (10 mcg), Gm (10 mcg), Mc (30 mcg), Nb (30 mcg), Pc (10 IU), Sm (25 mcg), Tc (30 mcg).

DISCUSSION

Bacterial plasmids are covalently linked to closed extra chromosomal DNA material inside the cell, and carry antimicrobial resistance encoding genes (Rosander *et al*., 2008). There has been a positive correlation between the number of plasmids and multi-drug resistance in *Escherichia coli*, isolated from poultry meat (Londhe *et al*., 2013).

In our study, we observed that the plasmid number was limited to one in most of the strains of CNS (78.4%), which were scattered over all the seven farms. However, its resistance pattern was different in different farms indicating the presence of different antibiotic resistance genes in different strains of CNS, bearing structurally similar plasmids (26083 bp).

We have isolated six species of CNS, out of which *S. xylosus* was the predominant species (43.2%), followed by *S. epidermidis* (29.6%). Krithiga *et al*. (2011) had reported 13 species of CNS microorganisms from cases of bovine clinical mastitis in Pondicherry. The predominant pathogen was *S. epidermidis* (23.33%), while the incidence of *S. xylosus* was 14.44 percent. It was just reverse in our study. Our results agreed with Soares *et al*. (2012). The number of species and their proportions might differ in different geographical locations, and thus in different farms.

The species of CNS displayed multidrug resistance. The magnitude was low (3.2%) in case of chloramphenicol, and very high (99.2%) in case of penicillin. The antibiotic resistance of different species of CNS, as observed in our study, was very high as compared to earlier reports (Luthje and Schwarz, 2006; Nunes *et al*., 2007; Waller *et al*., 2011).

CONCLUSION

Seven CNS strains were isolated from bovine SCM in our study Their resistance pattern was different in different farms. This is an indication that treatment in cases of bovine SCM should be undertaken after antbiogram analysis. The CNS strains showed multi-drug resistance, but there was least resistance to chloramphenicol. Plasmid-profile analysis gives an impression that antibiotic resistance is not linked with plasmid number. On the contrary, this property is possessed by specific plasmids. Thus, it might not be feasible to differentiate the antibiotic resistance property of CNS on the basis of plasmid profiling. However, plasmid profiling can be pursued as a useful epidemiological tool to demarcate geographical distribution of CNS linked bovine SCM.

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