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Cultural Isolation, Identification and Antibiotic Resistance of *Streptococcus agalactiae* from Bovine Sub-Clinical Mastitis Cases

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ABSTRACT

Streptococcus agalactiae causes sub-clinical mastitis in cattle which subsequently causes loss of milk production and also jeopardizes the quality of milk. The aim of this study was to isolate, identify and study the antibiogram of *Streptococcus agalactiae* prevalent in the nearby dairy farm of Anand Agricultural University, Anand. It was found that out of 47 cows and 107 udder quarters, 39 (82.97%) cows and 82 (76.63%) udder quarters were positive for sub-clinical mastitis by California Mastitis Test (CMT). On cultural isolation from milk samples of 82 sub-clinical mastitis positive quarters, 27 Streptococci isolates were confirmed by CAMP (Christie-Atkins-Munch-Peterson) test. All the 27 Streptococci isolates were confirmed as *Streptococcus agalactiae* by PCR using *Streptococcus agalactiae* specific primers (Sag 432/ Sag 1018) for the 16S rRNA. The antibiogram pattern of the *Streptococcus agalactiae* isolates was studied by Disc diffusion method for the seven antibiotics. It was found that the isolates were most sensitive for Gentamycin followed by Enrofloxacin, Ampicillin, Co-trimoxazole and Erythromycin. Least sensitivity was obtained for Tetracycline and Streptomycin.

HIGHLIGHTS

- From 82 sub-clinical mastitis positive quarters, 27 Streptococci isolates were obtained.
- Isolates were most sensitive for gentamycin followed by Enrofloxacin, Ampicillin, Co-trimoxazole and Erythromycin. Least sensitivity was obtained for Tetracycline and Streptomycin.

Keywords: Sub-clinical mastitis, antibiotic resistance, California mastitis test, *Streptococcus agalactiae*

Streptococcus agalactiae, the lone member of the Lancefield group B, is an important cause of chronic, contagious bovine mastitis. In case of sub-clinical mastitis, no visible abnormalities are found in bovine milk although milk yield and somatic cell count are changed in milk. The incidence of sub-clinical mastitis is higher than for clinical mastitis (Kabelitz *et al*., 2021). *Streptococcus agalactiae* also causes mastitis and invasive disease in camels and is an occasional cause of disease in dogs, cats, fish, and hamsters. Its presence is frequently associated with high somatic cell counts in milk and decreased milk yield (Martin *et al*., 2020). Unidentified carrier cows are responsible for spreading the pathogen to herd mates (Kumar and Yadav, 2012). *Streptococcus agalactiae* infections have major consequences for public health, because they may cause neurological problems in newborn humans and endometritis and sterility in the mothers. *Streptococcus agalactiae* may cause meningitis, septicemia, and prenatal inflammatory events associated with a high risk of periventricular leukomalacia (Stoll *et al.,* 2011). Strategies to prevent neonatal colonization and infection involve intrapartum antibiotic prophylaxis

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for all colonized mothers during labor and treatment of all colonized neonates (Ohlsson and Shah, 2009). *Streptococcus agalactiae* is an obligate parasite of the epithelium and tissues of ruminant mammary glands, and eradication of the organism from herds is, therefore, possible by identification of animals with mammary infection followed by treatment or culling.

Treatment with intramammary infusion of antibiotics is the main approach to deal with the *Streptococcus agalactiae* infection, and number of studies on *in vivo* and *in vitro* trials to assess the antibiotic sensitivity/resistant pattern have been documented. Therefore, the main aim of this study was to isolate the Group B *Streptococcus agalactaie* of bovine origin and to study its antibiotic sensitivity.

In the present study *Streptococcus agalactiae* were culturally isolated from the milk samples of sub-clinical mastitis positive animals and then the identification of these isolates was done by using gene specific primer followed by its antibiogram study.

MATERIALS AND METHODS

Milk collection and California Mastitis Test (CMT)

Milk samples from dairy farm near Anand Agricultural University, Anand were collected from 47 lactating cows after discarding first 2-3 streams of milk. Approximately 30 ml fore milk samples were collected from each quarter in a separate sterile screw-capped vial on three consecutive days during the evening milking for conducting CMT which was performed on fore milk samples as per the method described by Schalm and Noorlander (1957).

Cultural isolation

A heavy inoculum of thoroughly mixed quarter milk samples were inoculated on 5% sheep blood agar for primary bacterial isolation. The plates were incubated at 37°C for 24-48 h. Following the incubation, the plates were examined for bacterial growth and the morphological characteristics of bacterial colonies were recorded.

Identification of *Streptococcus*

The smear was prepared from the isolated culture on clean grease free microscopic glass slide and stained with Gram's staining method and observed under microscope. Smear revealing Gram positive cocci arranged in chains were presumptively considered to be Streptococci. A loopful of growth from a colony of the organism was emulsified on the surface of a glass slide in a suspension of 3 per cent KOH. The suspension was stirred with help of loop continuously for 60 s after which the loop was gently pulled from the suspension. The KOH test was only considered positive if stringing occurred within the first 30 s of mixing the bacteria in the KOH solution. Gram positive bacteria suspended in the KOH solution generally display no reaction i.e., absence of stringing. The presumptive Gram positive cocci were tested for catalase activity. Briefly, a drop of 3 per cent hydrogen peroxide (H_2O_2) was taken on a clean glass slide and the bacterial colony was picked up with the help of platinum inoculation loop and touched with it. The positive reaction was indicated by the formation of gas bubbles. A loopful of bacterial growth was rubbed on a sterile oxidase disc (Hi Media Ltd, Mumbai). Positive reaction was indicated with the development of deep purple blue or mauve colour within 10 s and no change in colour was considered as negative reaction. All the Streptococci isolates were tested by CAMP test as per the method of Sandholm (1995) with some modifications.

Molecular identification of Streptococcus

PCR was carried out for the identification of the Streptococcus isolates by using primer sag F 432 and sagR1086 as per Riffon *et al*. (2001) (Table 1).

Table 1: Details of primers for amplification of *sag* gene of *Str. agalactiae* employed in PCR

Primer	Sequences (5'- 3')	Target Gene	Size of amplified product (bp)
Sag (F)	CGT TGG TAG GAG TGG AAA AT	*sag*	586
Sag (R)	CTG CTC CGA AGA GAA AGC CT		

Antibiotic sensitivity test (ABST)

The ABST was performed as described earlier (Bauer *et al*., 1966). The antibiotic discs were obtained from

HiMedia Laboratories Ltd. Mumbai. Isolates were tested against commonly used antibiotics viz., Streptomycin (ST, 300 mcg), Tetracycline (T, 10 mcg), Co-trimoxazole (CO, 25 mcg), Enrofloxacin (En, 10 mcg), Erythromycin (E, 15 mcg) Gentamicin (G, 10 mcg) and Ampicillin (A, 10mcg).

RESULTS AND DISCUSSION

Detection of sub-clinical mastitis in dairy herd

The identification of pathogens causing mastitis is important for disease control and epidemiological studies. In a clinical laboratory, this is done using traditional microbiological methods. Although the microbial pathogens responsible for mastitis may be identified by conventional *in vitro* culture and biochemical tests, they are time consuming (3 to 7 days), laborious and not highly specific. Moreover, sub-clinically infected animals intermittently shed the bacteria that may yield no bacteria with milk culture (Phuektes *et al*., 2001). Therefore, identification of bacteria from sub-clinical mastitis cases and their antibiogram study is of utmost importance.

In the present study, out of 47 animals and 107 quarters positive for sub-clinical mastitis 39 (82.97%) cows and 82 (76.63%) udder quarters were positive by CMT. On bacteriological examination and growth on blood agar twenty seven (21.43%) isolates were identified as Streptococci. Earlier workers also used CMT and impregnated pH strip, either alone or in combination, for screening of sub-clinical mastitis in animals including Ali *et al*. (2008), Rady and Sayed (2009), Dubal *et al*. (2010), Elango *et al*. (2010), Varatanovic *et al*. (2010), Sindhu *et al*. (2011) and Tufani *et al*. (2011). Continuous usage of CMT and bacteriological methods timely detect presence of subclinical mastitis and give satisfactory results in prevention and therapy of mastitis, as well as improvement in amount and quality of milk (Varatanovic *et al*., 2010). The Somatic cell count (SCC) and CMT tests can be routinely used to screen herds for detection of sub-clinical mastitis. El-Balkemy *et al*. (1997) concluded that the CMT is still the superior screening diagnostic aid for subclinical mastitis, while bacteriological examination is still the most suitable, accurate and reliable method to confirm the causative organisms. Bacteriological sampling is not routinely feasible to identify sub-clinical mastitis in field conditions. Forester *et al*. (1983) reported that if CMT reactions score T, 1, 2 and 3, then the loss of quarter milk comes to 0.4 kg, 0.95 kg, 1.72 kg and 2.33 kg, respectively. In present study, quarter milk samples had CMT scores of 1, 2 or 3 which reflect daily loss of milk due to sub-clinical mastitis in dairy farms (Fig. 1).

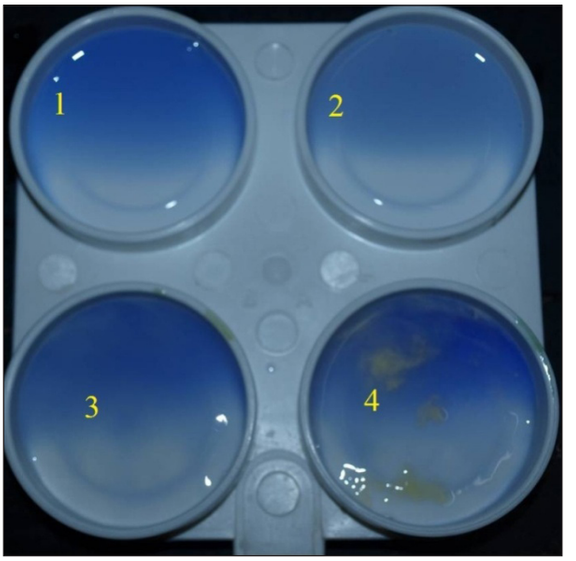

Fig. 1: Photograph showing results of California Mastitis Test 1-Negative (-), 2-Weak positive (1+), 3-Distinct positive (2+), 4-Strong positive (3+)

Identification of *Streptococcus agalactiae* from milk

Streptococci are Gram positive cocci in chain, negative by KOH test, catalase negative, oxidase negative and which fail to grow on MacConkey agar. The CAMP test, originally described by Christie *et al*. (1944) is often selected from several presumptive tests, because it requires minimal reagents and a simple inexpensive methodology. The CAMP test appears to be the most accurate and the most thoroughly evaluated (Facklam and Padula, 1974). *Str. agalactiae* elaborates factors which completely lyse the red blood cells already damaged by the β haemolysin producing *S. aureus* (Quinn *et al*., 1994). In this study, all the 27 Streptococci isolates were subjected for CAMP test and found to be strong CAMP test positive as indicated by production of arrowhead hemolysis confirming them as *Str. agalactiae* (Fig. 2, Table. 2).

Table 2: Identification of *Str. agalactiae* in cows with subclinical mastitis by biochemical tests, cultural isolation, CAMP test and PCR

Sl. No.	Animal no.	Quarter	KOH test	Gram's Stain	Catalase test	CAMP test	Haemo Lysis	Oxidase test	PCR result
1	1	HR	-	+	-	+	NH*	-	+
2	2	FL	-	+	-	+	NH	-	+
3	3	FR	-	+	-	+	NH	-	+
4	4	FL	-	+	-	+	NH	-	+
5	4	HR	-	+	-	+	NH	-	+
6	4	HL	-	+	-	+	NH	-	+
7	4	FR	-	+	-	+	NH	-	+
8	5	HR	-	+	-	+	NH	-	+
9	5	HL	-	+	-	+	NH	-	+
10	6	FL	-	+	-	+	NH	-	+
11	6	FR	-	+	-	+	NH	-	+
12	7	HL	-	+	-	+	NH	-	+
13	7	FL	-	+	-	+	NH	-	+
14	8	FL	-	+	-	+	NH	-	+
15	8	FR	-	+	-	+	NH	-	+
16	8	HL	-	+	-	+	NH	-	+
17	8	HR	-	+	-	+	NH	-	+
18	9	FL	-	+	-	+	NH	-	+
19	10	FR	-	+	-	+	NH	-	+
20	11	FL	-	+	-	+	NH	-	+
21	11	HL	-	+	-	+	NH	-	+
22	12	HL	-	+	-	+	NH	-	+
23	13	HL	-	+	-	+	NH	-	+
24	13	FL	-	+	-	+	NH	-	+
25	14	HR	-	+	-	+	NH	-	+
26	15	HR	-	+	-	+	NH	-	+
27	16	FL	-	+	-	+	NH	-	+

HR: Hind Right Quarter; HL: Hind Left quarter; FL: Fore Left quarter; FR: Fore Right Quarter; NH: Non haemolytic

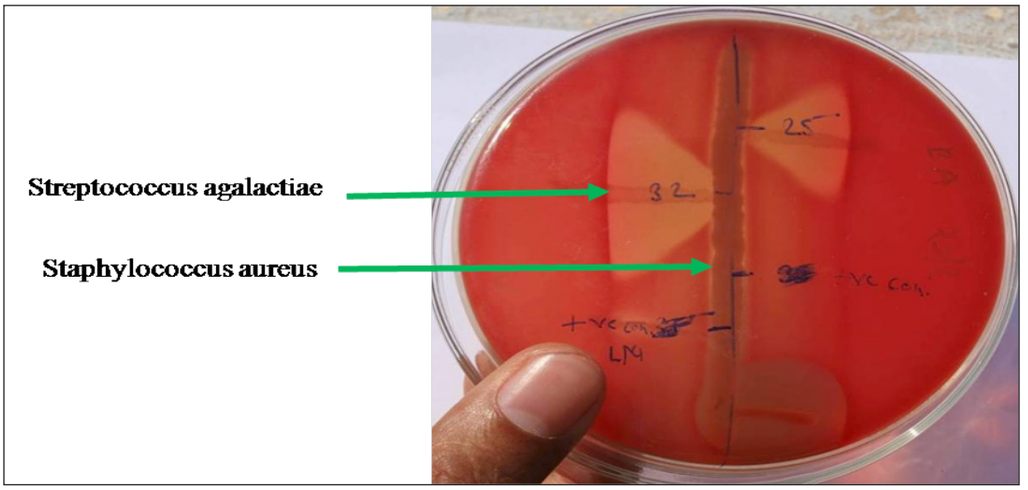

Fig. 2: Showing arrowhead hemolysis (arrow)-positive CAMP test for *Streptococcus agalactiae* with *Staphylococcus aureus* in sheep blood agar

Confirmation of culture isolates of *Streptococcus agalactiae* by PCR

In the present study, template DNA was obtained by boiling method which yielded good results in PCR. This technique proved to be very simple and rapid technique for template DNA preparation. On PCR amplification all the 27 streptococci isolates tested, could be identified as *Str. agalactiae* they yielded an expected amplification product of 586 bp (Fig. 3). The major disadvantage of PCR is due to excessive sensitivity, as minor contaminants in samples could lead to misdiagnosis. In addition, PCR cannot provide the information on antimicrobial sensitivity that is necessary for choosing drugs for treatment of mastitis cases. Due to presence of PCR inhibitors, detection of pathogens directly in milk samples requires its standardization. Phuektes *et al*. (2001) reported that when PCR was used to detect pathogens directly in milk samples, it was less sensitive than conventional culture. Looking to the advantage and disadvantage of PCR based identification of major bacterial pathogens, this part of the study was carried out after conventional cultural identification.

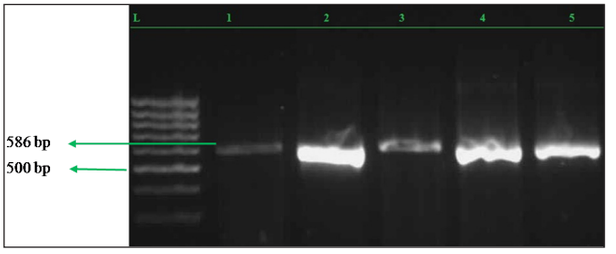

Fig. 3: Agarose gel electrophoresis of PCR amplified *Str. agalactiae* DNA product showing 586bp using primer set Sag 432/Sag 1018

Antibiogram of clinical isolates of *Streptococcus agalactiae*

Str. agalactiae infections in both humans and bovines are treated by administration of antibiotics. Penicillin is the drug of choice for treatment of both human and bovine *Str. Agalactiae* infections (Schrag *et al*., 2000). For penicillin-allergic individuals, erythromycin and clindamycin are recommended. The prevalence of resistance to erythromycin and clindamycin has been increasing in *Str. agalactiae* (Uh *et al*., 2001); resistance to erythromycin has been associated with human *Str. agalactiae* serotype III and V isolates (Lin *et al*., 2000). Extensive use of antibiotics in medicine and animal husbandry results in increased antibiotic resistance among bacterial populations.

In the present study, the pattern of antibiotic resistance of *Str. Agalactiae* isolates to seven antimicrobial agents were studied namely Streptomycin, Tetracycline, Erythromycin, Co-trimoxazole, Ampicillin, Enrofloxacin and Gentamicin. It was found that the isolates were most sensitive for Gentamycin followed by Enrofloxacin, Ampicillin, Co-trimoxazole and Erythromycin.

Streptococcus agalactiae isolates were found variably resistant to the antibiotics tested. Overall, higher percent of the isolates were resistant to Streptomycin (85.1%), followed by Tetracycline (55.5%), Erythromycin (33.3%), Co-trimoxazole (11.1%), Ampicillin (11.1%), Enrofloxacin (7.4%), and Gentamicin (3.7%) (Table 3).

Table 3: Over all resistance of *Str. agalactiae* isolates to antimicrobial agents

Antibiotic	Isolates					
	Sensitive		Intermediate		Resistant	
	Total	%	Total	%	Total	%
Streptomycin	2/27	7.4	2/27	7.4	23/27	85.1
Tetracycline	4/27	14.8	8/27	29.6	15/27	55.5
Erythromycin	18/27	66.6	0/27	0	9/27	33.3
Co-trimoxazole	23/27	85.1	1/27	3.7	3/27	11.1
Ampicillin	23/27	85.1	1/27	3.7	3/27	11.1
Enrofloxacin	25/27	92.5	0/27	0	2/27	7.4
Gentamicin	26/27	96.2	0/27	0	1/27	3.7

The highest resistance was found for Streptomycin (85.1%) where as lowest resistance was found for Gentamicin (3.7%). In case of Tetracycline, resistance was 55.5%, while 33.3% isolates were resistant to Erythromycin, where as for Co-trimoxazole and Ampicillin resistance was 11.1% each and 7.4% isolates were resistant for Enrofloxacin. Though antibiotics use has its advantages, the intensive and extensive use of antibiotics has led to the emergence of antimicrobial resistance.

In this study, 55.5% isolates were resistant to Tetracycline, which is in contrast to Duarte *et al*. (2004), who observed all isolates resistant to Tetracycline. Higher percentage of Tetracycline resistant isolates were observed by Aminov

et al. (2000) as 85.2%, Poyart *et al*. (2002) as 85%, Zeng *et al*. as (2005) 88% and Antoine *et al*. (2009) as 80%.

For Erythromycin, 33.3% *Str. agalactiae* isolates were observed as resistant, which is in contrast to Zeng *et al*. (2005), who observed 67% isolates resistant to Erythromycin. Erythromycin resistance to *Str. agalactiae* was also recorded by Culebras *et al*. (2002) 72%, Duarte *et al*. (2004) 10.5%, Zhao *et al*. (2007) 3% and Domelier *et al*. (2008) 8%.

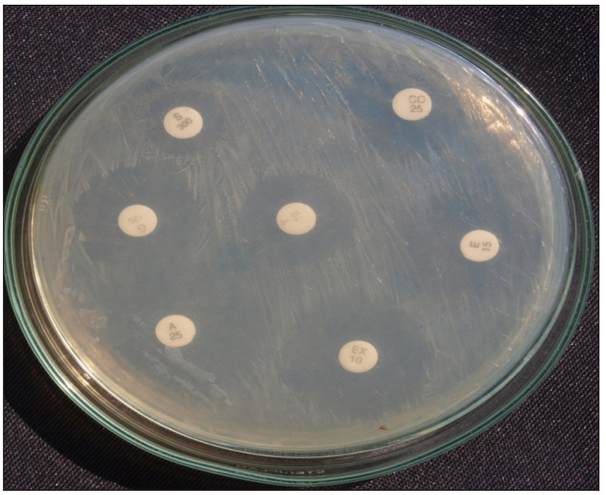

Fig. 4: Antibiogram of a *Streptococcus agalactiae* isolate

ST-Streptomycin, T-Tetracycline, Co-Trimoxazole, En-Enrofloxacin, E-Erythromycin, G- Gentamycin, and A- Ampicillin

CONCLUSION

The present study concludes that due to indiscriminate use of antibiotics in dairy animals the antibiotic resistance in the *Streptococcus agalactiae* is increasing which poses a threat to public health. Moreover, regular surveillance of dairy farm is required to monitor the antibiotic resistance level in the bacterial infections originating from milk of bovine origin.

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