The Pharma Innovation Journal 2023; SP-12(9): 134-136

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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(9): 134-136

www.thepharmajournal.com
Received: 19-06-2023
Accepted: 22-08-2023

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Isolation, identification and antibiogram pattern of bacterial isolates from subclinical mastitis samples of dairy cattle

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Abstract
The present study was conducted to determine the occurrence of subclinical mastitis (SCM) in pooled milking animal samples and to follow SCM cases in individual herds of selected villages. The California Mastitis Test (CMT) was proposed for evaluating SCM in pooled samples as well as individual animals in herds. All positive samples were cultured, isolated, and biochemically tested to determine the current state of the microorganisms involved in subclinical mastitis. ABST was performed on all of the isolates. The most common organisms isolated were coagulase positive Staphylococci. Bacterial isolates were very susceptible to Ceftriaxone but not to Penicillin-G.

Keywords: SCM, *Staphylococci*, CoPS, Sensitivity, Ceftriaxone, Penicillin-G

Introduction
Mastitis is a mammary gland inflammation that affects all domestic animals and is a serious concern for the dairy industry. Mastitis is a common disease in cattle in both developed and developing countries, and it has a large economic impact on dairy cow breeding since it occurs frequently and reduces milk output.
Mastitis can divided into per acute, acute and chronic based on the duration and clinical and subclinical based on the clinical signs exhibited by the animal (Kader *et al*., 2003) [7]. Approximately 75-80% of mastitis is subclinical, as evidenced by a considerably elevated leukocyte count in milk (Bradley 2002) [1].
Antimicrobial medications have been used in animals for many years for therapy, metaphylaxis, prophylaxis, and growth augmentation. The global use of antimicrobials in livestock production is predicted to expand by two-thirds between 2010 and 2030, with use doubling in Brazil, Russia, India, China, and South Africa (Van Boeckel, 2015) [21]. Furthermore, antibiotic-resistant bacteria are predicted to kill nearly 23,000 individuals in the United States each year (CDC, 2017) [2], with MRSA strains accounting for nearly half of these fatalities. In this context, the emergence and spread of antibiotic resistance continues to impede our ability to treat zoonotic illnesses, necessitating a complete understanding of resistance prevalence across zoonotic potential hosts.

Materials and Methods
The current study aimed to identify SCM in dairy cattle, as well as to investigate the present state of bacterial pathogens implicated in mastitis and the antibiotic resistance pattern in bacteria linked with subclinical mastitis in dairy cattle. CMT reagent (M/s Ruchi pesto chem (India) Pvt. Ltd. Pune)
Composition:
- Sodium hydroxide: 1.5% w/v
- Teepol 0.5% v/v
- Bromothymol blue 0.01% w/v

Milk samples were tested for mastitis using the California Mastitis test, as described by Quinn *et al*. (1999) [16].
CMT was used to detect SCM in pooled home milk samples. Positive samples were traced back to a specific residence, and 127 samples were taken from individual animals. Milk samples were taken during the evening milking. The first three foremilk streams were discarded. The teats were then meticulously cleansed with cotton and 70% ethanol.

15 mL of milk was collected aseptically in sterile vials. The samples were promptly delivered and refrigerated to the laboratory. S. aureus and other zoonotic potential bacteria, as well as the genetic causes of this resistance, in order to better monitor the risks to humans.

Milk samples were placed in nutritional broth and incubated at 37^0 degrees Celsius for 24 to 48 hours. Nutrient broth samples were scattered over Mannitol salt agar, brain heart infusion agar, MacConkey's agar plate, and Eosin Methylene blue agar selective media plates. These plates were incubated for 24 to 48 hours at 37 °C.

Gram's staining

Gram's staining of all the bacterial isolates was carried out as per the instructions mentioned in the kit.

Coagulase test

The slide coagulase test was performed on all Staphylococcus isolates collected. A drop of saline was placed on a clean grease-free slide, and a single isolated Staphylococcus colony from a BHI agar plate was mixed with the saline until a clear milky white suspension formed. For this, 50L of rabbit plasma was added and thoroughly mixed. Clot formation was considered positive for the Coagulase test.

Indole test

The presence of red or pink color in the alcohol layer after the addition of Kovac's reagent to the 48-hour-old tryptone broth culture was seen as a positive indicator of Indole formation.

Methyl red test

The presence of bright red color after adding of five drops of methyl red reagent to the 48-hour-old infected MR/VP medium was regarded as positive, whereas appearance of yellow color was regarded as negative.

Voges - Proskauer test

The appearence of red color within 15 minutes after addition of 0.6 mL of 5%- naphthol followed by 0.2 mL of 40% KOH to the 24 hour old inoculated MR/VP medium was regarded positive, whereas copper color was judged negative.

Citrate test

Development of blue color after 48 hours incubation of Simmon's citrate medium with the culture was considered positive.

Antibiotic sensitivity test of bacterial isolates

For bacteriological analysis, pure cultures from the selective media were added onto Nutrient broth and incubated at 37 ºC for six to eight hours to allow organisms to develop.

On Muller Hinton agar plate, bacterial isolates were tested for antimicrobial sensitivity using the disc diffusion method described by Cruickshank *et al*. (1975) [3].

Using a sterile cotton swab, the broth culture was placed to the surface of a Muller Hinton agar plate, and the plate was covered for 15 minutes at room temperature to allow the inoculum to dry.

Antimicrobial discs were carefully removed from their separate vials and placed 20 mm apart on the plates using flamed forceps. Plates were chilled for 15 minutes to allow antibiotic pre-diffusion before being incubated at 37 ºC for 18 to 24 hours.

The antibiotic sensitivity pattern of bacterial isolates to several antimicrobial discs was read by measuring the diameter of the zone of inhibition in millimeters using the manufacturers' chart.

Results and Discussion

In the current study among the 127 milk samples collected from subclinical mastitis cases, 105 samples were culturally positive for bacteria. A total of 164 bacterial isolates were recovered from these subclinical mastitis milk samples.

83 (50.61%) of the 164 isolates were Gram positive, whereas the remaining 81 (49.39%) were Gram negative. When cultured on selective media, the most common bacterial isolates recovered were Staphylococcus sp. (50.60%), Escherichia coli (37.80%), and Klebsiella sp. (11.58%).

According to this study, both gram positive and gram negative bacteria were involved in the development of SCM. Sumathi *et al*. (2008) [20], on the other hand, revealed that 65.33% were Gram positive and the remaining 34.67% were Gram negative.

The most common bacterial isolates collected in this study were Staphylococcus sp., Escherichia coli, and Klebsiella sp. Staphylococcus sp. appears to be the most common pathogen causing mastitis in cattle in this geographical area. Staphylococcus sp. was also found in the majority of mastitis cases described by Kayesh *et al*. (2014) [8] and Hegde *et al*. (2013) [5]. Similarly, Staphylococcus was identified as the most common pathogen by Khan and Muhammad (2005) [9], Sharma and Sindhu (2007) [18], Haftu *et al*. (2012) [4], Preethirani *et al*. (2015) [14], and Sanotharan *et al*. (2016) [18].

In the current study, most of the Coagulase positive *Staphylococcus* sp. isolates were sensitive to Ceftriaxone followed by Tetracycline, Doxycycline, Vancomycin, Streptomycin, Erythromycin and Tobramycin. Majority of isolates were resistant to Enrofloxacin, Rifampicin, Gentamicin, Kanamycin, Oxacillin, Penicillin- G and Cefoxitin.

The results are partially in accordance with the recordings of Preethirani *et al*. (2015) [14] who reported that CoPS were most resistant to Cefoxitin (100%) followed by Penicillin-G and Ceftriaxone/sulbactam. Further, they observed low resistance to Co-trimoxazole and Oxacillin and intermediate resistance to Enrofloxacin. They found all the CoPS susceptible to Chloramphenicol.

In contrary, Mohanty *et al.* (2013) [12] mentioned that the CoPS were sensitive to Enrofloxacin, Levofloxacin, Chloramphenicol and Gentamicin and resistant to Penicillin, Cefixime, Cephalexin, Amoxicillin and Oxytetracycline. Similarly, Mubarack *et al.* (2012) [13] opined that most of the *Staphylococcus* isolates were susceptible to Gentamicin, Cephalothin and Ampicillin and they were resistant to Streptomycin, Erythromycin and Tetracycline.

The variation in sensitivity of isolates depends on the frequency of antibiotics used in a particular area and this may be the reason for observation of variation in sensitivity reported by earlier few workers.

In the present study the isolates were sensitive to many antibiotics which could be recommended for treatment. However, the isolates were resistant to some of the commonly used antibiotics which warrants educating veterinarians about proper use of antibiotics for treatment of mastitis.

In the present study, majority of the *E. coli* isolates were sensitive to Tobramycin followed by Gentamicin, Ceftriaxone, Streptomycin, Doxycycline, Kanamycin, Cefoxitin and Tetracycline. All the isolates were resistant to

Enrofloxacin, Erythromycin, Penicillin- G, Rifampicin and Vancomycin.

The results of the present study are in partial agreement with that of Mohanty *et al.* (2013) [12] who reported that *E.coli* isolates were resistant to Penicillin-G and sensitive to Streptomycin and Ceftriaxone. In contrast, they also have reported that *E.coli* isolates were sensitive to Enrofloxacin. Similarly, the present study is in partial agreement with the reports of Preethirani *et al.* (2015) [14].

Poor hygienic conditions may contribute to a higher incidence of *E.coli* mastitis, as *E.coli* originates in the cow's surroundings and enters the udder via the teat canal (Mallikarjunaswamy and Murthy, 1997) [11].

In the present study, most of the *Klebsiella sp.* isolates exhibited sensitivity to Tobramycin and Gentamicin, followed by Ceftriaxone, Streptomycin, Doxycycline, Tetracycline, Cefoxitin, Kanamycin, Enrofloxacin, Oxacillin and Vancomycin. All the bacterial isolates were resistant to Erythromycin, Penicillin-G and Rifampicin.

The results of the study are in agreement with the findings of Kumar (2009) [10] who reported that *Klebsiella* isolates were sensitive to Ceftriaxone, Tetracycline, Streptomycin and Gentamicin.

The results of the study are also in partial agreement with that of Haftu *et al.* (2012) [4] who have reported that *Klebsiella* isolates were resistant to Erythromycin.

The majority of CoNS isolates in this investigation were sensitive to Ceftriaxone, followed by Tetracycline and Doxycycline. Erythromycin, Gentamicin, Kanamycin, Oxacillin, Penicillin-G, and Cefoxitin resistance was found in all isolates.

The current findings are in part consistent with the findings of Preethirani *et al*. (2015) [14], who found that CoNS were extremely resistant to Methicillin, Amoxycillin/sulbactam, and Penicillin-G but sensitive to Ceftriaxone/sulbactam and Cefotaxime. They have reported sensitivity to Cefoxitin and Gentamicin, on the other hand.

Similarly, Gentillini *et al.* (2002) [7] who also opined that CoNS isolates were resistant to Penicillin, Oxacillin and Erythromycin. In contrast they recorded that the CoNS isolates were sensitive to Gentamicin.

Conclusion

In the present study Coagulase positive Staphylococci were the most prevalent organisms isolated from SCM. Bacterial isolates were highly sensitive to Ceftriaxone and least sensitive to Penicillin-G. Since the isolates were resistant to most of the antibiotics, it is alarming that rational use of antibiotics is must in treating the mastitis.

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