Isolation and antibiogram of *Staphylococcus*, *Streptococcus* and *Escherichia coli* isolates from clinical and subclinical cases of bovine mastitis

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**Abstract**

**Aim:** The present study was aimed to isolate and evaluate the continuous change in the pattern of drug resistance showed by different mastitogenic organisms, isolated from clinical and subclinical cases of mastitis.

**Materials and Methods:** The study was carried out using 150 milk samples received from various clinical and subclinical cases, from which the causative organisms were isolated and subjected to *in vitro* antibiotic sensitivity test.

**Results:** The bacteriological analysis of the samples indicated the presence of both Gram positive and Gram negative organisms followed by isolation of isolates like *Staphylococcus*, *E. coli*, *Streptococcus*, *Bacillus*, *Corynebacterium*, *Listeria*, *Klebsiella*. The *in vitro* sensitivity of *Staphylococcus*, *E. coli* and *Streptococcus* isolates revealed that they were more sensitive towards newer antimicrobials like Levofloxacin and Enrofloxacin.

**Conclusion:** The prevalence of *Staphylococcus* was found to be maximum followed by *Streptococcus* and *E. coli* among the isolated organisms. Levofloxacin and Enrofloxacin were found to be most effective against the targeted isolates.

**Key words:** antibiotic sensitivity pattern, bovine, mastitis, milk.

**Introduction**

Mastitis is a multi-etiological complex disease which is characterized by physical, chemical and bacteriological changes in milk and pathological changes in glandular tissue of udder [1]. It is a devastating disease haunting the dairy industry worldwide and is a matter of great concern for leading milk producing country like India because of the losses incurred due to high morbidity, discarded milk, treatment costs and reduced milk production, thus drawing in more attention towards its treatment and control. It has also been found out that mastitis alone contributes to 70% of the losses during milk production which could have been avoided by means of prompt curative and control measures [2]. Particularly the Indian dairy industry suffers a monetary blow of over Rs. 6000 crores per year [3]. Apart from the economic losses, mastitis can have serious implications on public health. Mastitis which is mostly caused by the interaction of multiple pathogenic agents (primarily bacteria), can expose human beings to various morbidic organisms through infected milk, thus serving as a media for transmission of various zoonotic diseases like T.B, brucellosis, diphtheria, scarlet fever and Q fever [4]. Now there has also been increasing evidence of bidirectional transmission of Methicillin Resistant *Staphylococcus aureus* (MRSA) in humans and animals. Once exposed to MRSA, animals become reservoir of infection for human beings. So the increased antimicrobial resistance of the organisms in animals treated with antibiotics and their zoonotic transmission continues to be a matter of great concern globally [5]. The success of bovine mastitis therapy basically depends on the understanding of clinical presentation and antimicrobial susceptibility of the etiological agent, among various other factors [6]. One important reason for therapy failure in the management of mastitis could arise from various factors involving pathological changes in the udder, etiology, lower efficacy of antimicrobials, and improper veterinary services [7]. But above all, antimicrobial susceptibility testing, typically using a disk diffusion assay is often performed as part of the routine laboratory evaluation of milk samples with an attempt to provide guidelines for the treatment of mastitis, especially identification of the pathogens causing clinical and subclinical mastitis and thus forming a crucial part in development of mastitis control program. OIE has also recommended monitoring and observation of effectivity of antimicrobials towards pathogenic and commensual bacteria affecting animals [8].
Since the isolation of organism and choice of antimicrobial agent is often considered empirical in the management of mastitis, the present study was conducted to observe the sensitivity and resistance profile of mastitis causing isolates (particularly *Staphylococcus*, *Streptococcus* and *Escherichia coli*) within and around Bhubaneswar, against various commonly used antimicrobials.

**Materials and Methods**

Milk samples (n=150) from clinical and subclinical cases of mastitis were received by Department of Microbiology, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, from various regions of Bhubaneswar and areas around, for isolation, identification and antibiogram of the mastitogenic organisms during the period from 06.01.2009 to 05.08.2011. All the samples were showing positive result for Modified California Mastitis test. Each sample was taken by means of a sterile inoculating loop and introduced into liquid media (BHI broth) and incubated for substantial growth of microorganisms. Following growth in broth a loopful of culture was streaked onto muller hinton agar (MHA), eosin methylene blue (EMB) agar, mannitol salt agar (MSA), and blood agar and incubated for 24-48 hour at 37°C. The different colonies were marked and noted basing on their colony characteristics and growth on selective media. The colonies on blood agar were further analyzed basing on the hemolytic pattern. The morphological and staining characteristics of the organisms were determined by microscopic examination of Gram stained smears and a tentative analysis regarding the type of isolates was made. The isolates were identified according to cultural, morphological and biochemical characteristics complying with methods of Cruickshank [9]. The biochemical test and sugar fermentation properties for identification of isolates were conducted as per standard protocol [10, 11].

The *in vitro* antibiotic sensitivity pattern was studied by Kirby-Bauer disc diffusions method using 13 number of antibiotic discs (Himedia) such as Levofoxacin (5 mcg/disc), Enrofoxacin (5 mcg/disc), Penicillin-G (10 units/disc), Streptomycin (10 mcg/disc), Gentamicin (10 mcg/disc), Oxytetracycline (30 mcg/disc), Chloramphenicol (30 mcg/disc), Ceftriaxone tazobactum (30 mcg/disc), Cefixime (30 mcg/disc), Cephalexin (30 mcg/disc), Amoxycillin (10 mcg/disc), Ciprofoxacin (5 mcg/disc), Ofloxacin (5 mcg/disc). The inoculum was prepared by transferring 4-5 colonies from pure culture to BHI broth and incubated at 37°C for 6-8 hours until moderate turbidity developed. Then the inoculums turbidity was matched with standard 0.5 McFarland and diluted with NSS if required. The standardized inoculum was then applied onto the MHA agar plate by soaking with sterile cotton swab and allowed the inoculums to dry. The discs were then placed aseptically equidistant from each other on the surface of the agar plates. The plates were incubated at 37°C for 24-48 hours for development of inhibition zone. The diameters of zone of inhibition were then measured in millimeter and noted down [12]. The interpretation regarding the degree of susceptibility (resistant, moderate and highly sensitive) was made as per Clinical and Laboratory Standards Institute (formerly NCCLS) chart provided by the antibiotic disc manufacturer.

**Results and Discussion**

**Identification & isolation of organisms:** Out of the 150 samples processed, 6 are found to be sterile as no growth was observed in liquid and solid media, which may have been due to prior medication before collection of milk from the udder [13]. Bacteriological screening of the samples revealed 17 samples being pure cultures whereas the remaining 127 invariably showed the presence of mixed cultures, which may be
reported in an efficient way. On carrying out the antibiogram of these bacteria from milk samples has been previously the condition in day to day animal husbandry practices. But in the present study, the period of presence of Staphylococcus was found in the targeted isolates (2, 19, 20, 21). But in the present study, the period of presence of pure culture and mixed cultures was of result of lowered resistance of the udder [14]. The predominance of Staphylococcus isolates over Streptococcus in subclinical cases of mastitis has been highlighted in the study of many workers previously [17, 18]. Though the colonies picked up at random from the isolation plate and their morphological and staining characteristics. The pure culture revealed the high prevalence of due to secondary invasion by opportunist bacteria as a result of lowered resistance of the udder [14]. The presence of pure culture and mixed cultures was ascertained by the different types of colonies growing on selective media (MSA, EMB and blood agar), hemolytic pattern on blood agar, biochemical test and sugar fermentation properties whereas the inference regarding other isolates was made as per the methods followed above except for the growth on selective media. Isolation of these bacteria from milk samples has been previously reported [2, 14, 15, 16]. Of the 144 samples, Staphylococcus was found in 47%, whereas Streptococcus and E. coli in 32% & 21% of the samples respectively [Table-1]. The predominance of Staphylococcus isolates over Streptococcus has been cited in the work of several other workers [2, 19, 20, 21]. But in the present study, the period of investigation showed a high prevalence of Staphylococcus followed by Streptococcus, and E. coli. Similar findings were also observed by several workers in different parts of the globe [18, 22, 23, 24]. However, GITAU et al. [25] have found the prevalence of Streptococcus species to be highest in mastitis.

Antibiogram of the isolates: It was revealed during the isolation and identification that Staphylococcus, Streptococci and E. coli are the major pathogens responsible for clinical and sub clinical cases of mastitis and study of their response to various antibiotics will help in the management and control of the condition in day to day animal husbandry practices in an efficient way. On carrying out the antibiogram of the targeted isolates (Staphylococcus spp., Streptococcus spp., Proteus spp., Corynebacterium spp. and Bacillus spp. along with few samples positive for Listeria spp. and Klebsiella spp. in addition to the organisms isolated from pure culture. The conclusion regarding the identity of Staphylococcus, Streptococcus and E. coli was made on basis of their motility, staining, cellular morphology, characteristic growth on selective media (MSA, EMB and blood agar), hemolytic pattern on blood agar, biochemical test and sugar fermentation properties whereas the inference regarding other isolates was made as per the methods followed above except for the growth on selective media. Isolation of these bacteria from milk samples has been previously reported [2, 14, 15, 16]. Of the 144 samples, Staphylococcus was found in 47%, whereas Streptococcus and E. coli in 32% & 21% of the samples respectively [Table-1]. The predominance of Staphylococcus isolates over Streptococcus in subclinical cases of mastitis has been highlighted in the study of many workers previously [17, 18]. Though high prevalence of Staphylococcus spp. followed by E. coli has been cited in the work of several other workers [2, 19, 20, 21]. But in the present study, the period of investigation showed a high prevalence of Staphylococcus followed by Streptococcus, and E. coli. Similar findings were also observed by several workers in different parts of the globe [18, 22, 23, 24]. However, GITAU et al. [25] have found the prevalence of Streptococcus species to be highest in mastitis.

**Table-1. Frequency of isolation of Staphylococcus, Streptococcus and E. coli from clinical samples of mastitis**

| Sr. no. | Bacterial species      | No. of isolates out of 144 non sterile samples | Approx. percentage |
|--------|------------------------|-----------------------------------------------|-------------------|
| 1      | Staphylococcus spp.    | 68                                            | 47                |
| 2      | Streptococcus spp.     | 46                                            | 32                |
| 3      | E. coli                | 30                                            | 21                |
| Total  |                        | 144                                           |                   |

**Table-2. Overall antimicrobial susceptibility profile**

| Antibiotic        | Highly sensitive (%) | Moderately sensitive (%) | Resistant (%) |
|-------------------|----------------------|--------------------------|--------------|
| Chloramphenicol   | 77.7                 | 11.8                     | 10.4         |
| Amoxycillin       | 6.94                 | 38.19                    | 54.86        |
| Enrofloxacillin   | 87.5                 | 10.4                     | 2.08         |
| Streptomycin      | 24.30                | 46.52                    | 29.16        |
| Levofloxacin      | 90.27                | 6.94                     | 2.77         |
| Pencillin-G       | 0                    | 21.52                    | 78.47        |
| Gentamicin        | 65.97                | 31.94                    | 2.08         |
| Ciprofloxacin     | 80.55                | 16.66                    | 2.77         |
| Ofloxacin         | 51.38                | 34.02                    | 14.58        |
| Cephalaxin        | 8.72                 | 24.30                    | 65.97        |
| Oxytetracycline   | 15.97                | 51.98                    | 32.63        |
| Cefixime          | 4.86                 | 29.86                    | 65.27        |
| Ceftriaxone+tazobactum | 61.11              | 24.30                    | 14.58        |

**Table-3. Antibiogram of Staphylococcus isolates**

| Antibiotic        | Highly sensitive (%) | Moderately sensitive (%) | Resistant (%) |
|-------------------|----------------------|--------------------------|--------------|
| Chloramphenicol   | 77.94                | 10.29                    | 5.55         |
| Amoxycillin       | 0                    | 44.11                    | 55.88        |
| Enrofloxacillin   | 88.23                | 10.29                    | 1.47         |
| Streptomycin      | 14.70                | 54.41                    | 30.88        |
| Levofloxacin      | 88.23                | 8.82                     | 2.94         |
| Pencillin-G       | 0                    | 17.64                    | 82.35        |
| Gentamicin        | 73.52                | 26.47                    | 0            |
| Ciprofloxacin     | 75                    | 25                       | 0            |
| Ofloxacin         | 55.88                | 33.82                    | 10.29        |
| Cephalaxin        | 22.05                | 22.05                    | 55.88        |
| Oxytetracycline   | 11.76                | 55.88                    | 32.35        |
| Cefixime          | 0                    | 33.82                    | 66.17        |
| Ceftriaxone+tazobactum | 66.17              | 22.05                    | 11.76        |

**Table-4. Antibiogram of Streptococcus isolates**

| Antibiotic        | Highly sensitive (%) | Moderately sensitive (%) | Resistant (%) |
|-------------------|----------------------|--------------------------|--------------|
| Chloramphenicol   | 69.56                | 15.21                    | 15.21        |
| Amoxycillin       | 2.17                 | 41.30                    | 56.52        |
| Enrofloxacillin   | 89.13                | 10.86                    | 0            |
| Streptomycin      | 26.08                | 32.60                    | 45.65        |
| Levofloxacin      | 89.13                | 6.52                     | 2.17         |
| Pencillin-G       | 0                    | 39.13                    | 60.86        |
| Gentamicin        | 65.21                | 34.78                    | 0            |
| Ciprofloxacin     | 86.95                | 15.04                    | 0            |
| Ofloxacin         | 43.47                | 32.60                    | 34.78        |
| Cephalaxin        | 13.04                | 21.73                    | 65.22        |
| Oxytetracycline   | 17.39                | 71.73                    | 10.86        |
| Cefixime          | 0                    | 32.60                    | 67.39        |
| Ceftriaxone+tazobactum | 60.86              | 28.26                    | 10.86        |

**Table-5. Antibiogram of E. coli isolates**

| Antibiotic        | Highly sensitive (%) | Moderately sensitive (%) | Resistant (%) |
|-------------------|----------------------|--------------------------|--------------|
| Chloramphenicol   | 90                    | 10                       | 0            |
| Amoxycillin       | 30                    | 20                       | 50           |
| Enrofloxacillin   | 83.33                | 10                       | 6.66         |
| Streptomycin      | 43.33                | 50                       | 6.66         |
| Levofloxacin      | 96.66                | 3.33                     | 0            |
| Pencillin-g       | 0                    | 3.33                     | 96.66        |
| Gentamicin        | 50                    | 40                       | 10           |
| Ciprofloxacin     | 83.33                | 3.33                     | 13.33        |
| Ofloxacin         | 53.33                | 36.66                    | 10           |
| Cephalaxin        | 10                    | 33.33                    | 56.66        |
| Oxytetracycline   | 23.33                | 10                       | 66.66        |
| Cefixime          | 0                    | 16.66                    | 83.33        |
| Ceftriaxone+tazobactum | 50                | 23.33                    | 26.66        |
Antibiogram of *Staphylococcus* isolates: *Staphylococcus* is the most important and prevalent mastitogenic organism occurring globally, including India [2]. In case of studies relating to Staphylococcus, Unakal et al. [5] have found that the highest numbers of *Staphylococcus aureus* isolates were susceptible to Ceftiraxone followed by Cefotaxime, Methicillin, Ciprofloxacin, Erythromycin, Amikacin, Gentamicin, Amoxyccillin, Ampicillin, whereas the lowest susceptibility was shown in Penicillin-G. However, the present study contrastingly showed a decreasing trend of sensitivity of *Staphylococcus* towards Ceftiraxone combination of drugs [Table-3], but was well in agreement with other reports that stated newer drugs like Levofloxacin, Enrofloxacin, Chloramphenicol and Gentamicin were effective in treatment of Staphylococcal mastitis [26].

Antibiogram of *Streptococcus* isolates: *Streptococcus* being second highest in the list following the current study is responsible for chronic contagious bovine mastitis and understanding its susceptibility towards antimicrobials is very essential. Jain et al. [27] found the higher susceptibility of *Streptococcus* isolates towards Enrofloxacin and Gentamicin, whereas Mahanests et al. [4] reported higher susceptibility to Tetracycline and Chloramphenicol. Similar concordance has been found in the current study where it was found that the *Streptococcus* isolates were most susceptible to Enrofloxacin, Levofloxacin and Ciprofloxacin [Table-4].

Antibiogram of *E. coli* isolates: The incidence of *E. coli* mastitis may have been due to poor hygienic conditions, as *E. coli* originates from the cow’s environment and infect the udder via the teat canal [28]. In the current study it was observed that the *E. coli* isolates were most sensitive toward Levofloxacin and Chloramphenicol followed by Enrofloxacin, Ciprofloxacin, Ofloxacin and Gentamicin [Table-5]. However this was in contrast to the study of Moges et al. [22] who found *E. coli* to be highly susceptible to Chloramphenicol and Clindamycin. Similarly Khan et al. [29] showed that *E. coli* were sensitive to Gentamicin, Ciprofloxacin, Chloramphenicol, Cephalothin, Amoxyccillin and Oxytetracycline.

The increased resistance to these therapeutic agents can be attributed to injudicious use of antibiotics in the mastitis cases [4, 14, 17, 20, 21, 26, 30] along with negligence towards the bacteriological examination of the suspected milk. Though species, regional and managemental variations leads to variation of results in the antibiogram study but also the preferences of antibiotics used by various workers leads to inaccurate correlation among the studies relating to the sensitivity of mastitogenic isolates towards antibiotics. But even with the study of findings of various workers, the present study draws the conclusion that newer drugs like Levofloxacin and Enrofloxacin are very much effective for treatment of mastitis. But development of resistance in bacteria against these agents cannot be ruled out in the near future if injudicious use of antibiotics continues further.

**Conclusion**

Development of resistance among bacteria is a matter of great concern in the field of veterinary medicine especially when the organisms renders an animal unproductive, thus posing a direct threat to country’s economy. So as a well planned therapy is very essential in control and cure of mastitis, conducting an antibiogram profile of the mastitogenic organisms provides a clear insight towards management of mastitis. In the present study, the prevalence of *Staphylococcus* was found to be maximum followed by *Streptococcus* and *E. coli* among the isolated organisms. These three isolates showed high sensitivity towards antibiotics like Levofloxacin and Enrofloxacin. Considering the clinical and economic aspect of the disease, awareness is required among farmers and clinicians for preventing the drugs from falling into the resistant category. Accurate diagnosis of mastitis, isolation and identification of causal organisms, establishment of correct *in vitro* antibiogram along with proper food and hygienic status of the animal is very much essential to prevent mastitis cases and economic losses thereof.

**Authors’ contributions**

NNM, LNS and HKP have conceived, planned and designed the study. PD, SSP, SR have isolated, carried out the antibiogram profile, analysed and kept a due record of the data. Manuscript was framed and drafted by NNM, LNS under the guidance of HKP. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Aim: The present study was aimed to isolate and evaluate the continuous change in the pattern of drug resistance showed by different mastitogenic organisms, isolated from clinical and subclinical cases of mastitis. Escherichia coli isolates from clinical and subclinical cases of bovine mastitis. Nihar Nalini Mohanty, Priyaranjan Das, Shaswati Subhadarsini Pany, Laxmi Narayan Sarangi, Siddharth Ranabijuli and Hemant Kumar Panda. Subclinical cases of mastitis has been highlighted in the. on the isolation plate and their morphological and. study of many workers previously [17, 18]. Streptococcus and E. coli among the isolated organisms. of drugs [Table-3], but was well in agreement with. These three isolates showed high sensitivity towards. In this study, the anti-biofilm effect of GSE was investigated against biofilm-forming strains of Staphylococcus aureus and Escherichia coli. The GSE minimum inhibitory concentration (MIC) for S. aureus and E. coli were 25 Î¼g/ml and 250 Î¼g/ml, respectively. To investigate biofilm inhibition and degradation effect, crystal violet assay and stainless steel were used. Biofilm formation rates of four strains (S. aureus 7, S. aureus 8, E. coli ATCC 25922, and E. coli O157:H4 FRIK 125) were 55.8%, 70.2%, 55.4%, and 20.6% at 1/2 Â— MIC of GSE, respectively. Biochemical characterization and antibiogram of staphylococcal microorganisms associated with subclinical mastitis in lactating crossbred cows. Animal Science Reporter, 8 (4): 123-129. Radostits, O. M., Gay, C. C., Hinchcliff, K. W. and Constable, P. D. 2007. Saei, H. D. 2012. Coa types and Antimicrobial resistance profile of Staphylococcus aureus isolates from cases of bovine mastitis. Comparative Clinical Pathology, 21 (3): 1-7. Schlam, O.W. and Noorland, D.O. 1957.