

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 morbid 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 commensal bacteria affecting animals [8].

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Figure-1. From top left to right: Sugar fermentation test to identify *Staphylococcus* spp, Sugar fermentation test to identify *E.coli*, Haemolytic colonies of *Streptococci* spp on blood agar, Antibiotic sensitivity test of the isolates.

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

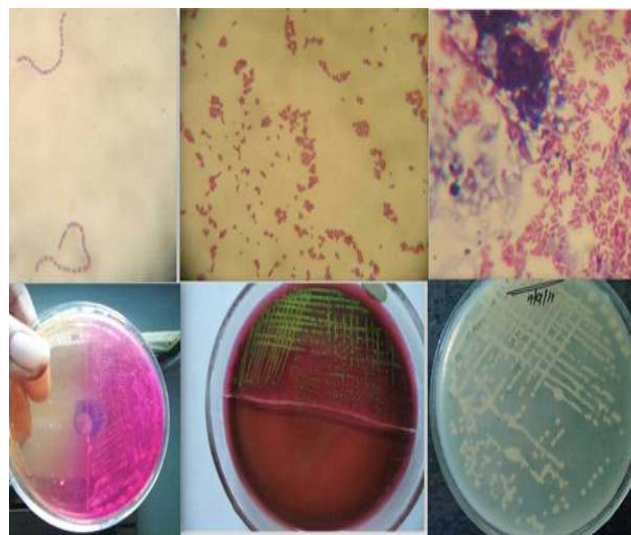


Figure-2. From top left to right: *Streptococcus* spp, *Staphylococcus* spp, Gram negative bacilli, colony growth on mannitol salt agar, Metallic sheen on EMB agar by *E.coli* isolates, colonies on MHA agar.

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 Levofloxacin (5 mcg/disc), Enrofloxacin (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 tazobactam (30 mcg/disc), Cefixime (30 mcg/disc), Cephalexin (30 mcg/disc), Amoxycillin (10 mcg/disc), Ciprofloxacin (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 inoculum 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 inoculum 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

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	<i>Staphylococcus</i> spp.	68	47
2	<i>Streptococcus</i> spp.	46	32
3	<i>E. coli</i>	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
Enrofloxacin	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
Cephalexin	9.72	24.30	65.97
Oxytetracycline	15.97	51.38	32.63
Cefixime	4.86	29.86	65.27
Ceftriaxone+tazobactam	61.11	24.30	14.58

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
Enrofloxacin	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	13.04	0
Ofloxacin	43.47	32.60	34.78
Cephalexin	13.04	21.73	56.52
Oxytetracycline	17.39	71.73	10.86
Cefixime	0	32.60	67.39
Ceftriaxone+tazobactam	60.86	28.26	10.86

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 the isolation plate and their morphological and staining characteristics. The pure culture revealed the presence of *Staphylococcus*, *Streptococcus* and *E. coli* whereas the colonies picked up at random from the mixed cultures showed the presence of *Pseudomonas* 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

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
Enrofloxacin	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
Cephalexin	22.05	22.05	55.88
Oxytetracycline	11.76	55.88	32.35
Cefixime	0	33.82	66.17
Ceftriaxone+tazobactam	66.17	22.05	11.76

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

Antibiotic	Highly sensitive (%)	Moderately sensitive (%)	Resistant (%)
Chloramphenicol	90	10	0
Amoxycillin	30	20	50
Enrofloxacin	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
Cephalexin	10	33.33	56.66
Oxytetracycline	23.33	10	66.66
Cefixime	0	16.66	83.33
Ceftriaxone+tazobactam	50	23.33	26.66

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.

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., *E. coli*.) it was found that most of the isolates were showing high sensitivity towards Chloramphenicol, Enrofloxacin, Levofloxacin, Ciprofloxacin while resistance pattern was more evident for some classical antibiotics like Penicillin-G, Cephalexin, and Amoxicillin. The sensitivity pattern for Streptomycin, Ceftriaxone, Gentamicin and Oxytetracycline showed an alarming indication of increasing resistance among the bacterial organisms to these therapeutic agents [Table-2]. This study was in close agreement with many workers [22, 26]. Farooq et al. [23] during their study period have found that Norfloxacin, Gentamicin and Chloramphenicol were the most effective drugs where as Amoxicillin, Kanamycin, Sulphamethaxazole, Streptomycin and Penicillin-G showed lesser effectiveness against mastitis isolates. Harini et al. [20] screened milk samples and found bacterial isolates highly sensitive for Ciprofloxacin, Ofloxacin, Enrofloxacin, Gentamicin and Chloramphenicol, with pattern of resistance towards Colistin, Neomycin, Streptomycin, Penicillin and Tetracycline. Similarly Jeykumar et al. [21] found Enrofloxacin to be most effective among the antibiotics whereas Sumathi et al. [2] reported Gentamicin to be most effective among the antimicrobials used in study.

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 Ceftriaxone followed by Cefotaxime, Methicillin, Ciprofloxacin, Erythromycin, Amikacin, Gentamicin, Amoxicillin, Ampicillin, whereas the lowest susceptibility was shown in Penicillin-G. However the present study contrastingly showed a decreasing trend of sensitivity of *Staphylococcus* towards Ceftriaxone 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 Mahantesh 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, Amoxicillin 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 managerial 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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