MICROBIOLOGICAL AND ANTIBIOGRAM STUDY OF BACTERIAL PATHOGENS ASSOCIATED WITH BOVINE MASTITIS IN AND AROUND MEERUT

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ABSTRACT

A rise in the incidence of disease in a herd results in multiplied use of antimicrobials, which in turn increases the presence of antibiotic residues in milk and increased bacterial resistance to antimicrobials. Continued use of antibiotics in the remedy and prevention of diseases of dairy cows always needs to be scrutinized. With this background, we carried out the present study to screen the major pathogens of bovine mastitis circulating in and around Meerut, Uttar Pradesh, India and their sensitivity to frequently used antibiotics. 30 milk samples suspected for mastitis based on clinical manifestations were collected and processed for bacterial isolation, identification and culture sensitivity test. Among the isolates, 14 (46.67%) were Gram-positive bacteria, 11 (36.67 %) Gram negative and 5 (16.67 %) were mixed infection. The isolated mastitis dweller bacteria were *Staphylococcus* spp. (46.67 %), *E. coli* (36.67 %), and mixed infection spp. (16.67 %). The studies of *in vitro* antibiogram revealed gentamicin to be the most effective drug (93.34 %), followed by enrofloxacin (66.67 %), cefotaxime+clavulanic acid (63.34 %), ampicillin+sulbactam (60.00 %), chloramphenicol (60.00 %), amoxicillin+sulbactam (53.34 %), colistin (46.67 %), ciprofloxacin (40.00 %), oxytetracycline (33.34 %), streptomycin (33.34 %), amoxicillin + clavulanic acid (13.34 %) and ampicillin/cloxacillin (10.00 %) against the bacterial isolates from mastitis milk. These findings suggest bacterial resistance against commonly used advanced drugs and combination of drugs. Thus, it can help to develop guidelines for practitioners in the choice of the most appropriate antibiotic. The outcomes of study contribute to risk assessment of anti-microbial resistance (AMR) and provide a standard baseline for setting up and assessing control measures and structuring strategies to constrain AMR.

Keywords: Mastitis, bovine, prevalence, antibiogram, antimicrobial resistance

INTRODUCTION

Bovine mastitis is the inflammation of the mammary gland that has over 130 different isolated causative agents from mastitis milk followed by physical, chemical and bacteriological modifications in milk and glandular tissue[1]. Perceived worldwide as likely the costliest diseases influencing dairy herds, it reduces the milk yield and quality of milk and increases rate of culling and veterinary cost. It has been assessed that the mastitis alone can cause almost 70 % of all avoidable misfortunes occurring during milk production. It is assumed that one significant reason behind treatment failure is unpredictable utilization of antimicrobial agents without testing *in vitro* sensitivity of causal organisms[2]. Since the extensive utilization of antibiotics can prompt resistance[3], AMR occurrence must be consciously controlled to guide prudent prescription[4]. In regard of the habitat of pathogen, mastitis is assessed as: (A) contagious mastitis, which is occur by contagious bacteria dwelling on the skin of the teat and inside the udder, transmitted starting with one cow to next by milking (e.g., *Staphylococcus aureus* or *Streptococcus agalactiae*) and (B) environmental mastitis, which is brought about by environmental pathogens commonly found in the dairy animal vicinity such as bedding, manure, soil, and feed[5]. (e.g., *Escherichia coli*, *Streptococcus uberis*, *Klebsiella* sp.). In India, annual economic loss to dairy industry due

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to subclinical mastitis and clinical mastitis is estimated to be Rs. 4151.1 and Rs. 3014.4 crores[6], respectively. The long-time utilization of antimicrobials in the treatment of mastitis has accentuated further problems of antimicrobial resistant strains, therefore there is persistent worry about treatment failure and about the resistant strains entering the food chain. Treatment failures additionally lead to longer times of infectivity, which increase the number of infected cattle moving in the farm and in this way expose the entire herd to the risk of contracting a resistant strain of infection[7]. There is a need for new antimicrobials to replace over-used conventional antibiotics[8]. Therefore, continual consideration has been given by the analysts to discover the correct antibiotic agents to treat and control mastitis doing antibiotic sensitivity test. Keeping these points in view, we conducted this study with the objective to identify the major pathogens associated with mastitis and to select a suitable antibiotic for treatment.

MATERIALS AND METHODS

Collection of milk samples

During 3 months (January 2020- March 2020), thirty milk samples from clinical mastitis cases were collected from various dairy farms in and around Meerut and from cases that were presented in the veterinary clinical complex, College of Veterinary and Animal Sciences, Meerut. We had collected aseptically milk samples from the infected farm animals in sterile vials. Before sample collection, the udder was thoroughly washed with potassium permanganate solution (1:1000) and wiped with clean cloth to allow dry and the teats were mopped with 70 % ethyl alcohol. Relevant information about the farm, breed and history of individual animals were recorded.

Media reagents and chemicals

The media and chemicals were obtained from Hi-Media, Mumbai (India) and prepared in the laboratory as per the standard procedures[9].

Isolation and identification of isolates

A total of 30 milk samples were inoculated on blood agar (BA), brain heart infusion agar (BHI) and MacConkeys lactose agar (MLA) plates for bacterial isolation. The inoculated plates were incubated aerobically at 37 ^{o}C for 24-48 h. The typical colonies were sub-cultured in a selective broth and subjected to various tests viz., Gram reaction, oxidase, catalase, IMViC, motility and growth on TSI slant for biochemical characteristics, as per the method of Quinn[10].

In vitro antimicrobial susceptibility testing

All the bacterial isolate was analyzed against twelve different antimicrobial discs (Hi-Media, Mumbai, India), namely–amoxyclav (30 mcg), amoxicillin+sulbactum (30/15 mcg), ampicillin+cloxacillin (10 mcg), ampicillin+sulbactum (10/10 mcg), cefotaxime+clavulanic acid (30/10 mcg), ciprofloxacin (5 mcg), chloramphenicol (30 mcg), colistin (10 mcg), enrofloxacin (10 mcg), gentamicin (10 mcg), oxytetracycline (30 mcg) and streptomycin (10 mcg). The disc diffusion method as described by Bauer[11] was employed and the interpretation was made as per the zone size interpretation chart provided by the manufacturer of discs.

Statistical analysis

The experimental data generated from the *in vitro* tests were calculated for all variables in terms of frequencies and proportions and associations between variables were determined by Chi-square test using SPSS (20.0).

RESULTS AND DISCUSSION

Gross examination of the milk samples

During the sample collection, the results obtained from the visual examination of the milk drawn in the collection tube are represented in Table I. The analysis performed of the pooled milk per animal on the viscosity and watery condition and presence of blood showed that a majority of the samples (40 % to 60 %) contained flakes / clots. About, 06 % to 08 % of the samples exhibited watery condition while no blood was observed in any samples.

Table I: Visual examination of milk samples from mastitis

Group	No. of animals	Flakes/Clot		Watery	
		N	%	n	%
Cow breeds	20	12	60	8	40
Buffalo breeds	10	4	40	6	60
Total	30	16	53.3	14	46.7

Isolation of bacteria

Out of 30 mastitic milk samples, 24 (82.0 %) samples showed bacterial growth and 06 (18.0 %) samples were negative for any bacterial growth. Out of 30 isolates, 14 (46.67 %) were Gram positive, 11 (36.67 %) Gram negative and 05 (16.67 %) showed mixed infection (Fig. 1).

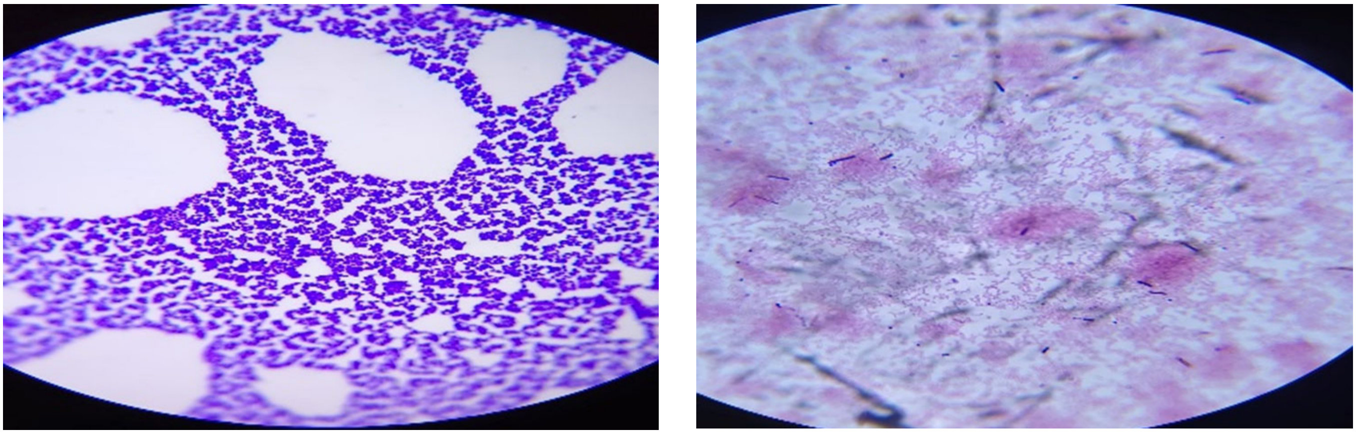

Fig. 1: Microscopic appearance of bacteria culture of *Staphylococcus spp.* and *E. coli* by Gram's staining

The investigation showed that the major predominant pathogens related with bovine mastitis in and around Meerut was *Staphylococcus spp.* (46.67 %), trailed by *E. coli* (36.67 %), and mixed infection (16.67 %). The prevalence of mastitic agents in and around Meerut is depicted in Table II.

Table II: Prevalence of bacterial pathogens in and around meerut (n = 30)

Isolate	No. of Positive samples	Per cent (%)
Staphylococcus spp.	14	46.67
E. coli	11	36.67
Mixed infection (Gram positive & Gram-negative bacilli)	5	16.67

In vitro antimicrobial susceptibility testing

The *in vitro* antibiogram studies (Table III) of the bacterial isolates from mastitis milk revealed gentamicin to be the most effective drug (93.34 %), followed by enrofloxacin (66.67 %), cefotaxime+clavulanic acid (63.34 %), ampicillin+sulbactam (60.00 %), chloramphenicol (60.00 %), amoxicillin+sulbactam (53.34 %), colistin (46.67 %), ciprofloxacin (40.00 %), oxytetracycline (33.34 %), streptomycin (33.34 %), amoxyclave (13.34 %) and ampicillin/cloxacillin (10.00 %).

Statistical analysis

The calculated X^2 had a level of significance less than 0.05 (Table IV). It can be concluded that different microbe's sample in variances have significant differences in sensitivity against various antibiotics used in *in vitro* study.

Table III: Comparative assessment of antibiotics against the bacterial pathogens and their overall per cent (%)

Antibiotic	*Staphylococcus* Spp.		*E. coli*		Mixed infection		Overall Percent (%)
	n=14	%	n=11	%	n=5	%	n=30
Amoxyclav	4	28.57	1	9.09	0	0	16.67
Amoxycillin/sulbactam	9	64.28	4	36.36	3	60	53.33
Ampicillin/cloxacillin	3	21.42	0	0	0	0	10.00
Ampicillin/sulbactam	10	71.42	3	27.27	1	20	46.67
Cefotaxime/clavulanic acid	13	92.85	5	45.45	3	60	70.00
Ciprofloxacin	14	100.00	7	63.64	5	100	86.67
Chloramphenicol	6	42.85	7	63.64	3	60	53.33
Colistin	9	64.28	7	63.64	1	20	56.67
Enrofloxacin	13	92.85	7	63.64	5	100	83.33
Gentamicin	14	100.00	9	81.82	5	100	93.33
Oxytetracycline	6	42.85	2	18.18	1	20	30.00
Streptomycin	8	57.14	9	81.82	5	100	73.33

Table IV: Statistical data analysis of antibiotics

Antibiotic		Sensitive	Resistance	Total
Amoxyclave	Count	5	25	30
	%within Antibiotic	16.7%	83.3%	100.0%
Amoxycillin/sulbactam	Count	16	14	30
	%within Antibiotics	53.3%	46.7%	100.0%
Ampicillin/cloxacillin	Count	3	27	30
	%within Antibiotics	10.0%	90.0%	100.0%
Ampicillin/sulbactum	Count	14	16	30
	%within Antibiotics	46.7%	53.3%	100.0%
Cefotaxime/clavulanic acid	Count	21	9	30
	%within Antibiotics	70.0%	30.0%	100.0%
Ciprofloxacin	Count	21	9	30
	%within Antibiotic	70.0%	30.0%	100.0%
Chloramphenicol	Count	16	14	30
	%within Antibiotic	53.3%	46.7%	100.0%
Colistin	Count	17	13	30
	%within Antibiotic	56.7%	43.3%	100.0%
Enrofloxacin	Count	25	5	30
	%within Antibiotic	83.3%	16.7%	100.0%
Gentamicin	Count	28	2	30
	%within Antibiotic	93.3%	6.7%	100.0%
Oxytetracycline	Count	9	21	30
	%within Antibiotic	30.0%	70.0%	100.0%
Streptomycin	Count	22	8	30
	%within Antibiotic	73.3%	26.7%	100.0%
Total	Count	202	158	360
	%within Antibiotics	56.1%	43.9%	100.0%

Chi-Square value is 97.681 (P=0)

DISCUSSION

In this examination, the mastitic agents had been isolated from 82 % cases, while no growth was obvious in 1 %. The failure of pathogens to grow *in vitro* in samples may be because of premedication of the animals with antibiotics, non-bacterial causes and the type of media which do not help the growth of whole range of bacteria related with mastitis. The study revealed that *Staphylococcus spp.* was the major etiological agent of causing mastitis with high prevalence. The next predominant isolate was *E. coli* followed by the mixed infection (Gram- negative bacilli and Gram-positive bacilli), which is in accordance with previous reports[12-14]. The results were also in consonance with the work of Verma[15] et al., who reported the higher prevalence of *Staphylococcus spp.* and *E. coli* from the cases of mastitic in and around Meerut and Sumathi[16] *et al.*, who found higher percentage of *E. coli* and *S. aureus* from clinical mastitis cases of dairy cattle in and nearby place in Bangalore. The details from other parts of the country[17-19] also show the maximum prevalence of *Staphylococci* followed by other mastitogenic microorganisms. Cheng[20] et al., reported 541 isolates of the 5 most common species, *S. aureus*, non-aureus *Staphylococci*, *Streptococcus* species, *Klebsiella* species, and *E. coli*, from bovine clinical mastitis on forty-five dairy farms in ten provinces of China. Prevalence of multidrug resistance was 27 %. An exceptionally wide distribution of minimum inhibitory concentrations was screened in all isolates, including *S. aureus* isolates, which were resistant to penicillin. Prevalence of resistance to both *E. coli* and *Klebsiella* spp. was high to amoxicillin/clavulanate potassium, followed by tetracycline. Hawariazmi and Fowzi[21] detailed that *S. aureus* (40.60 %) and coliform (26.10 %) were the chief etiological pathogens which were liable for clinical mastitis. They also reported the incidence of *Proteus* sp. (1.40 %), *Pseudomonas* sp. (4.30 %), mixed (7.30 %) and other (5.80 %) in clinical mastitis and these review helps the findings of the existing study. The higher incidence of *Staphylococci* shows unhygienic milking practices, as this pathogen is primarily spread during milking through milker's hands.

The bovine mammary gland can be a significant reservoir of enterotoxigenic strains of *S. aureus* whereas predominance of *E. coli* reflects bad hygienic practices in dairies as these organisms originate from the cow's environment and infect the udder through the teat canal. Contamination of end of the teat is a significant predisposing factor being cause of environmental mastitis[22]. The *in vitro* antibiogram profile of the bacterial isolates from mastitis milk revealed gentamicin to be most effective drug (93.34 %), followed by enrofloxacin (66.67 %), cefotaxime+clavulanic acid (63.34 %), ampicillin+sulbactam (60.00 %), chloramphenicol (60.00 %) and amoxicillin+sulbactam (53.34 %). All antimicrobial use in the herd may influence the resistance of *E. coli* isolates by expanding these antimicrobial agents in the dairy condition. The frequency of resistance *Staphylococcus* spp. mastitis was higher, which may be because of uncontrolled utilization of antibiotics and intramammary preparations containing combinations and broad-spectrum antibiotics[23]. The Chi-square score trend and indicates the highly significant value 97.681 (P=0). Indiscriminate use of antibiotics and intramammary preparations by the owner without the instruction of the veterinarian is also attributed to be one reason for increasing incidence of these strains. Fazel[24] et al., reported that 430 clinical mastitis samples were collected from 14 dairy herds in five different cities and in 70 *E. coli* were isolated. Most of isolates were resistant to lincomicin and streptomycin, whereas sulfa-trimethoprim had the least resistance rate. Iqbal[25] et al., have reported gentamicin, enrofloxacin and norfloxacin as best effective drugs among the 12 antibiotics tested *in vitro*. Higher efficacy of gentamicin, enrofloxacin and ciprofloxacin seen in the area of study has also been reported by Sumathi[16]. Gentamicin has been demonstrated as the drug of choice in this study. Few workers found maximum sensitivity of mastitic agents to gentamicin, enrofloxacin[26,27] and chloramphenicol[28] and much less sensitivity to ampicillin and cloxacillin. Unpredictable and frequent use of these antibiotics in animals could be the reason for their ineffectiveness towards mastitic bacteria. Edward[29] also suggested a possible advancement of resistance from prolonged and indiscriminate usage of certain antimicrobials.

CONCLUSION

It is very important to execute a systemic utilization of an antibiotic susceptibility test preceding the utilization of antibiotics in both treatment and prevention of intra-mammary infections. These findings also highlight the significance of considering both resistance and any temporal variation to characterize the AMR and estimate its potential threat. It is submitted that for success of the treatment, the antibiotic sensitivity test assumes a significant role.

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