ANTIBIOGRAM ANALYSIS OF BOVINE MASTITIS-CAUSING BACTERIA ISOLATED FROM NEARBY FARMS OF LAHORE

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Abstract:
Bovine mastitis has become major issue globally. This research is performed for investigating the current status of clinical mastitis among dairy cattle of farms of Lahore. The prevalence of mastitis was assessed by the results of the bacteriological evaluation of milk samples collected from clinical mastitis cases from farms in and nearby Lahore. Mastitic cattle and buffalos from farms nearby Lahore, Pakistan, have a high prevalence of multidrug-resistant bacteria. The main bacterial pathogen linked to mastitis was Staphylococcus aureus, followed by E.coli and S. dysgalactiae. This indicates that unhygienic and unsatisfactory management practices are being followed on the nearby farms. About 42 different mastitogenic bacteria including Staphylococcus aureus (28), Escherichia coli (12), and Streptococcus dysgalactiae (2) were isolated from the 47 confirmed clinical cases of mastitis affected cows and were further processed for antibiotic resistance profiling. The prevalence of major pathogens isolated was prevalence of 66.67%, 28.57%, and 4.76%, respectively. The results are similar to the earlier report of S.aureus (34%), E.coli (19%), and Streptococci spp. (9%) by Australia. Antibiogram analysis were also performed for these isolates shows that Gentamicin, Ciprofloxacin and Meropenem can be used as efficient antimicrobial agents for the treatment of bovine mastitis. This study has shown that microbiological and antibiogram analysis is very important for treatment and control of the mastitis.
Keywords: Bovine mastitis, AST, Antibiogram, microbiological analysis

Introduction:

Bovine mastitis has become a major issue globally. It is an inflammatory response of the udder tissue of cattle and buffalos resulted from some injury or microbial illness. It has become extremely complex and the costliest disease in India. It affects 50% of the herd population1. It has been estimated that the mastitis alone can cause approximately 70% of all avoidable losses incurred during milk production. It is believed to be the disease that causes the biggest financial damage to the dairy sector because of lower yield and poor milk quality. Bovine mastitis is thought to have a $147 average annual cost per animal for failure overall. Approximately 11 to 18 percent of the gross margin per animal each year is contributed by milk production losses and 2.Injury to mammary tissue, which decreases milk production, is responsible for 70% of the total losses 3.

Overuse of antibiotics without performing AST is the main reason for treatment failure(2). This practice results in economic losses and development of AMR(antimicrobial resistance)(3). The current study is intended to understand the antimicrobial resistance pattern (AMR) among the three most common mastitogenic bacteria including S.aureus, Streptococcus spp, and E.coli isolated from mastitic milk samples of infected bovines from various dairy farms of Lahore.

Material and Methods

1. **Collection of bacterial isolates:** For investigation, 60 milk samples were collected from clinical mastitis cases from different dairy farms located nearby Lahore. All information about the farm, breed and demographic history of each animal was.recorded.
2. **Media reagents and chemicals**: Different bacteriological grade media, such as mannitol salt agar, MacConkey agar, blood agar, and nutrient agar, were prepared in accordance with the manufacturer's instructions. The pH was adjusted using solutions of 10% NaOH and 10% HCL and were sterilized by autoclaving and then they were poured into glass Petri plates already sterilized by dry heat using hot air oven.
3. **Identification of isolates:** S.aureus, E.coli, and S.dysgalactiae were streaked on mannitol salt agar, MacConkey agar, and blood agar, respectively. After being incubated at 37°C for 24-48 hours, the plates were assessed for growth as well as morphological features, including colony size, shape, color, and hemolytic traits. To obtain pure cultures, suspected colonies were chosen, and sub-cultured on nutrient agar. And then gram staining and biochemical tests(catalase and oxidase test) are performed for identification.
4. A**ntimicrobial susceptibility testing:** Minimal inhibition concentration (MIC) values of each isolated bacterium were analyzed. A panel of antimicrobials was prepared for each bacterial species. Amoxicillin-clavulanate potassium/ Augmentin (20/10µg), penicillin G (10units), gentamicin (10 µg), ciprofloxacin (5µg), clindamycin (2µg), chloramphenicol (30µg), and erythromycin (15µg) were selected for Staphylococcus aureus; augmentin (20/10µg), ampicillin (10µg), aztreonam (30µg), gentamicin (10µg), chloramphenicol (30µg), cefixime (5µg), ciprofloxacin (5µg), tetracycline (30µg), meropenem (10µg), and Co-trimoxazole (1.25/23.75µg), were used for Escherichia coli; and penicillin G (10units), vancomycin (30µg), erythromycin (15µg), tetracycline (30µg), chloramphenicol (30µg), ampicillin (10µg), linezolid (30µg), and clindamycin (2µg) were used for S.dysgalactiae. The zones of inhibition were then measured in mm and compared to CLSI standards for interpretation.
5. Broth Microdilution Method:
   The guidelines of the Clinical and Laboratory Standards Institute were followed for performing the broth microdilution method (Patel, Patel, Weinstein, Richter, & Eliopoulos, 2017).

I. *Preparation of Cation Adjusted Mueller Hinton Broth (CAMHB):*

21g of the dehydrated powder of Mueller Hinton broth (HIMEDIA) was dissolved in 1000mL of distilled water, pH was adjusted at 7.3±0.1 at 25° and sterilization by autoclaving. A tiny amount of MHB medium was used to dissolve 20–25 mg of $CaCl_2$ and 10–12.5 mg of $MgCl_2$, separately. The solutions were then passed through a 0.2µm syringe filter and added to the medium. After the sterility test, it was used for the broth microdilution procedure.

II. ***Preparation of Stock Solution:***
10mg of the lyophilized powder of vancomycin (Bosck Pharmaceuticals) was dissolved in 10mL of sterile distilled water and sterilized by passing through a 0.2µm syringe filter. It was then used for the preparation of working solution.

III. ***Preparation of Working Solution:***
10mL of cation-adjusted Mueller Hinton broth (CAMHB) was taken in a sterile falcon tube and 25.6µL of the broth was replaced with the stock solution, according to the formula $C_1V_1=C_2V_2$. The resulting solution had 256µg of vancomycin in 10mL of CAMHB medium which was then used for making dilutions.

IV. ***Preparation of Bacterial Inoculum:***
Three to four bacterial colonies from purified cultures were selected and transferred to 10mL of sterile normal saline in a test tube. 100µL from the suspension was transferred to a 96-wells microtiter plate and the OD was adjusted to 0.08-0.1 using an ELISA reader. Perform 10 fold dilution until the bacterial suspension became equivalent to 105 CFU/mL.

V. ***Performance of Broth Microdilution Method:***
200µL of the working solution was added to the first well of each row in a 96-wells microtiter plate. 100µL of cation-adjusted Mueller Hinton broth (CAMHB) was then added to the second well up till the last well of each row. 2-fold serial dilution was performed well till the 10th. Finally, 100µL of the bacterial suspension was added up to the eleventh well and zero day OD was measured at 630nm wavelength with the help of an ELISA reader. The 11th and 12th wells served as the respective positive and negative controls. After 24 hours of incubation at 37°C, the day one OD value was obtained, and ODnet was computed. The least concentration of vancomycin showing no obvious growth was taken as the minimum inhibitory concentration of the antibiotic. Finally, the results were interpreted as sensitive, resistant, or intermediate using the CLSI standards.

Results:

From 60 milk samples, 75 bacterial isolates were isolated. Out of 60 samples, pure cultures were isolated from 21 samples (35%). Out. OllLf which 6 were gram-positive and 15 were gram-negative organisms and the remaining 39 (65%) yielded mixed cultures. Of the 75 isolates, 49 (65.33%) were gram- positive and remaining 26 (34.67%) were gram-negative. The predominant bacterial isolates recovered were Staphylococcus aureus (24%) and Escherichia coli (20%) followed by Staphylococcus epidermidis(16%), Streptococcus sp. (16%), Klebsiella sp. (10.67%), Bacillus sp. (5.33%), Corynebacterium sp. (4%), Proteus sp. (2.66%) and Pseudomonas sp. (1.33%). The frequency of isolation of different bacterial species from clinical mastitis cases is depicted in Table1. The high prevalence of Staphylococcus species followed by E. coli in the present study is in accordance with work of several other workers.

Staphylococci are the most important and prevalent mastitis causing organism globally, including

India. Higher incidence of E. coli mastitis may be due to poor hygienic conditions, as E. coli originates from the cow's environment and infect the udder via the teat canal10. The in vitro antibiogram studies of the bacterial isolates from mastitis milk revealed gentamicin to be most effective drug (90%) followed by enrofloxacin (88%),ciprofloxacin (85%),chloramphenicol (75%), tetracycline (60%), colistin (57%),neomycin (50%), nitrofurantoin (50%), furazolidone (50%), cephalexin (47%), penicillin (45%), streptomycin (35%) and sulphadiazine (30%). Gentamicin, enrofloxacin, ciprofloxacin and chloramphenicol are newer chemotherapeutic agents and are less commonly used for treatment of mastitis in the area of study resulting in higher efficacy of these drugs. Gentamicin proved to be the drug of choice in this study. Similar antibiogram pattern were repor ted by other workers also12,13,14. number of isolates showed resistance to cephalexin, penicillin, streptomycin and sulphadiazine. Indiscriminate and frequent use of these antibiotics in animals could be the reason for their ineffectiveness against bacterial isolates. Production of plasmids mediated beta-lactamase enzymes is supposed to be mainly responsible for resistance to penicillin. Since streptomycin has been extensively used along with penicillin for treating mastitis; it may have led to the development of high resistance in bacteria against this antibiotic. Whereas the resistance to sulphadiazine could be due to either low affinity of the enzyme that uses the p-amino benzoic acid during folic acid synthesis or use of preformed folic acid from surroundings.

Results and Discussion:

Different mastitogenic bacteria including Staphylococcus aureus, Escherichia coli, and Streptococcus dysgalactiae were isolated and cultured on various selective and differential media including mannitol salt agar, MacConkey agar, eosin methylene blue agar, and blood agar to obtain pure cultures. They were further confirmed through various phenotypic tests including Gram staining, catalase test, and oxidase tests to re-confirm the isolates. Antibiotic sensitivity assay was then performed through broth microdilution and disc diffusion assays to obtain an antibiogram.

Macroscopic Analysis:

Various selective and differential media were used for the macroscopic analysis of the isolates like mannitol salt agar was used for Staphylococcus aureus; MacConkey agar and eosin methylene blue agar were used for Escherichia coli and; blood agar was used for Streptococcus dysgalactiae. The colony morphology of the isolates is mentioned in table 4.1

Table 4.1: Colony Morphology of *S.aureus, E.coli, and S.dysgalactiae*

Sr.No.	Bacterial Isolate	Medium	Size	Color	Shape
1.	*Staphylococcus aureus*	Mannitol Salt Agar	Small to Medium	Yellow	Round
2.	*Streptococcus dysgalactiae*	Blood Agar	Pinpoint	White (Alpha Hemolytic)	Round
3.	*Escherichia coli*	MacConkey Agar	Small	Pink	Round
4.	*Escherichia coli*	Eosin Methylene Blue Agar	Medium	Green Metallic Sheen	Round

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Table 4.2: Microscopic characteristics of *S.aureus, E.coli, and S.dysgalactiae*

Sr.No.	Bacterial Isolate	Gram staining	Shape	Arrangement
1	*Staphylococcus aureus*	*G +ve*	*Cocci*	*Clusters*
2	*Streptococcus dysgalactiae*	*G +ve*	*Cocci*	*Chains*
3	*Escherichia coli*	*G -ve*	*Bacilli*	*Single*

Table 4.3: MDR Pattern of *S.aureus, E.coli, and S.dysgalactiae*

Sr.No.	Bacteria	Number of Isolates	Resistance to Antibiotics N (%)			Multidrug Resistant Bacteria (%)
			1 antibiotic	2 antibiotics	>2 antibiotics	
1	*Staphylococcus aureus*	28	3 (10.7%)	12 (42.9%)	13 (46.4%)	89.3
2	*Escherichia coli*	12	0 (0%)	0 (0%)	12 (100%)	100
3	*Streptococcus dysgalactiae*	2	0 (0%)	0 (0%)	2 (100%)	100

Sr.No.	Antibiotic	Concentration (µg)	Antibiotic Sensitivity Pattern		
			S	I	R
1	Augmentin	20/10	10.7%	0%	89.3%
2	Penicillin G	10 units	21.4%	0%	78.6%
3	Gentamicin	10	92.9%	0%	7.1%
4	Ciprofloxacin	5	89.3%	10.7%	0%
5	Clindamycin	2	42.9%	25%	32.1%
6	Chloramphenicol	30	71.4%	17.9%	10.7%
7	Erythromycin	15	46.4%	39.3%	14.3%

Table 4.4: Antibiotic sensitivity Pattern of *S.aureus*

Table 4.5: Antibiotic Susceptibility Pattern of *Streptococcus dysgalactiae*

Sr.No.	Antibiotic	Concentra-tion (µg)	Antibiotic Sensitivity Pattern		
			S	I	R
1	Augmentin	20/10	0%	0%	100%
2	Ampicillin	10	0%	0%	100%
3	Aztreonam	30	66.7%	0%	33.3%
4	Gentamicin	10	66.6%	16.7%	16.7%
5	Chloramphenicol	30	50%	16.7%	33.3%
6	Cefixime	5	25%	8.3%	66.7%
7	Ciprofloxacin	5	66.7%	0%	33.3%
8	Tetracycline	30	0%	0%	100%
9	Meropenem	10	91.7%	8.3%	0%
10	Co-trimoxazole	1.25/23.75	50%	8.3%	41.7%

Table 4.6: Antibiotic Susceptibility Pattern of *E.coli*

Sr.No.	Antibiotic	Concentra-tion (µg)	Antibiotic Sensitivity Pattern		
			S	I	R
1	Penicillin G	10units	50%	0%	50%
2	Vancomycin	30	100%	0%	0%
3	Erythromycin	15	100%	0%	0%
4	Tetracycline	30	0%	0%	100%
5	Chloramphenicol	30	50%	50%	0%
6	Ampicillin	10	0%	0%	100%
7	Linezolid	30	100%	0%	0%
8	Clindamycin	2	50%	0%	50%

In the current study, S.aureus, E.coli, and S.dysgalactiae were shown to be the main cause of bovine mastitis among the microbes obtained for the study with prevalence of 66.67%, 28.57%, and 4.76%, respectively. Since bacterial infection is the main cause of bovine mastitis, antimicrobial therapy is frequently used for its treatment. But antimicrobial resistance (AMR) has been linked to low cure rates(7). According to the World Health Organization (WHO), antimicrobial resistance is accelerated by the misuse and overuse of antimicrobials(4).

In the study, over 78% of the Staphylococcus aureus were resistant to penicillin G (natural penicillin) while 21% of them were sensitive to it(14). Augmentin is also a β-lactam antibiotic that consists of amoxicillin along with clavulanate, a β-lactamase inhibitor. In the current study, over 89.28% of S.aureus were resistant to augmentin and 10.71% of them were sensitive to it. However, E.coli showed 100% resistance toward augmentin. Ampicillin, an aminopenicillin, is semi-synthetic northwest Pakistan that 86.1% of the mastitogenic bacteria had developed resistance to ampicillin (15). In this study, over 100 percent of E.coli and S.dysgalactiae were found resistant to ampicillin.

Aztreonam is a monobactam that interferes in the synthesis of bacterial cell wall by binding to an enzyme, called transpeptidase just like penicillin. In this study, about 66.7% of E.coli were found susceptible to aztreonam. Meropenem is a carbapenem and has the same mechanism of action as other β-lactam antibiotics. It has also shown good results against mastitogenic bacteria with a susceptibility rate of 91.7%. This demonstrates that aztreonam and meropenem can be really helpful in the treatment of bovine mastitis caused by E.coli.

Cefixime is a third-generation cephalosporin. Over 66.7% of E.coli were found resistant to cefixime antibiotic making it an ineffective way of managing bovine mastitis. Many other antibiotics like vancomycin are also used for the treatment of mastitis in bovines. In December 2016, Vancomycin Resistant S.aureus (VRSA) was reported in the milk of bovines and caprines. This was the first report of VRSA in food animals, despite the fact that the bacteria is frequently detected in humans. The detection of VRSA in milk is a huge concern because it could further threaten the health of its consumers.

In the research conducted in India, about two (0.73%) out of 274 isolates of S.aureus exhibited vancomycin resistance with MICvan of 16µg/mL. While five isolates (1.83%) had shown intermediate response toward vancomycin with MICvan of 8µg/mL(18).In this study, a total of ten S.aureus isolates were checked for vancomycin resistance. All (100%) had shown sensitivity toward vancomycin with MICvan of 2 and 1µg/mL. There was not a single isolate that responded to vancomycin in an intermediate or resistance ranges. However, S.dysgalactiae was evaluated for vancomycin resistance and found 100% susceptible to it. In 2021, a study conducted in Pakistan showed that 77.7% of mastitogenic bacteria were resistant to erythromycin(15). While in this study, only 14.3% of S.aureus were found resistant to erythromycin. In the study, about 92.9% of S.aureus, and 66.6% of E.coli were susceptible to gentamicin. These results are similar to that of India (71.2%) (16), Poland (69.9%) (17),and northwest Pakistan (91.5%) (15). However, because of intrinsic resistance, aminoglycosides are not the preferred antimicrobials for the treatment of Streptococcal mastitis.

Tetracycline is a protein synthesis inhibitor. All the isolates of E.coli and S.dysgalactiae were found resistant to tetracycline in the study. Ciprofloxacin is a fluoroquinolon and a high proportion of S.aureus (89.3%), and E.coli isolates (66.7%) were found susceptible to ciprofloxacin.

Table 4.7: Mean Zone of Inhibitions and Minimum Inhibitory Concentration by *Staphylococcus aureus, Escherichia coli,* and *Streptococcus dysgalactiae*

Sr.No.	Bacteria	Antibiotic	ZOI and MIC (Mean)
1	*Staphylococcus aureus*	Penicillin G	18.54
		Augmentin	8.07
		Erythromycin	22.11
		Clindamycin	18.07
		Gentamicin	24.46
		Ciprofloxacin	26.5
		Chloramphenicol	25.36
		Vancomycin	15.20
2	*Escherichia coli*	Ampicillin	3.92
		Augmentin	1.00
		Cefixime	11.42
		Meropenem	29.08
		Aztreonam	23.75
		Gentamicin	18.09
		Tetracycline	2.17
		Ciprofloxacin	23.00
		Co-trimoxazole	14.83
		Chloramphenicol	16.00
3	*Streptococcus dysgalactiae*	Penicillin G	22.5
		Ampicillin	0.00
		Vancomycin	19.5
		Erythromycin	21.5
		Clindamycin	9.5
		Tetracycline	0.00
		Chloramphenicol	21.5
		Linezolid	26.5

Co-trimoxazole is a combination of sulphonamide and trimethoprim. In the study, about 12 isolates of E.coli were tested against co-trimoxazole. Five (41.7%) out of 12 isolates were resistant to the antibiotic while only one isolate (8.3%) gave an intermediate response to it (2).Chloramphenicol binds to bacterial ribosomes, shows very good results so far with a susceptibility rate of 84.1% against mastitogens (15). Also, it was found that 71.4% of S.aureus, 50% of E.coli, and 50% of S.dysgalactiae were susceptible to chloramphenicol in the current study. Linezolid belongs to the class of oxazolidinones, the study showed 100% susceptibility for this antibiotic against S.dysgalactiae.

The MDR (Multidrug Resistance) trend mostly followed by the isolates include Augmentin-Ampicillin-Cefixime-Tetracycline-Ciprofloxacin-Cotrimoxazle-Chloramphenicol for E.coli; Penicillin G-Augmentin-Clindamycin-Vancomycin for S.aureus; and Ampicillin-Tetracycline for S.dysgalactiae. This trend is quite similar to the Penicillin-Augmentin-Vancomycin-Enrofloxacin-Tetracycline MDR trend for S.aureus; Augmentin-Tetracycline-Kanamycin-Cefquinome trend for E.coli; and Penicillin-Augmentin-Tetracycline trend for Streptococcus species reported by China (17).

The data was analyzed with the help of descriptive analysis by SPSS (version 20.0) and the results were compared with the standards of the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST), which provided the results for sensitive, intermediate, and resistant isolates, based on all mean values. According to the study, all the bacterial isolates were sensitive, intermediate, and resistant for the antibiotics. All the isolates were sensitive for gentamicin and ciprofloxacin while E.coli was also susceptible to the meropenem antibiotic. The results of the mean zone of inhibitions and mean minimum inhibitory concentration for the isolates is mentioned in table 4.7.

Mastitis therapy typically takes longer than expected because the illness recurs and the bacteria that cause it are difficult to eradicate even with a variety of antimicrobials. According to the World Health Organization a post-antibiotic era, far from being an apocalyptic fantasy, in which typical illnesses and minor wounds can kill is actually a very plausible possibility for the twenty-first century (16). So, in order to develop effective control measures and suitable therapeutic procedures, it is crucial to have knowledge of the prevalence of bovine mastitis and its causative agents.

Conclusion:

This study comes to the conclusion that mastitic cattle and buffalos from farms nearby Lahore, Pakistan, have a high prevalence of multidrug-resistant bacteria. The main bacterial pathogen linked to mastitis was Staphylococcus aureus, followed by E.coli and S. dysgalactiae. This indicates that unhygienic and unsatisfactory management practices are being followed on the nearby farms. Moreover, on the basis of the antimicrobial susceptibility profiling of the isolates, gentamicin, ciprofloxacin and meropenem can be used as efficient antimicrobial agents for the treatment of bovine mastitis.

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