Indian J. Vet. Med. Vol. 42, No. 1, 2022 pp. 9-14

Research Articles

Antibiogram of *Staphylococcus aureus* isolated from mastitic milk samples of cows in Thrissur District

Krupa Rose Jose[1] and K. Vijayakumar[2]*

[1]PhD. scholar, [2] Professor and Head, Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India

Abstract

Antimicrobial resistance (AMR) among *Staphylococcus aureus* (*S. aureus*), a primary contagious pathogen that is consistently being identified from bovine mastitic milk imposes a significant therapeutic and economic burden in the treatment of mastitis and undermines the success of antimicrobial chemotherapy. Hence, the present study was conducted to investigate the antimicrobial susceptibility profile of *S. aureus* isolated from mastitic dairy cows of Thrissur district, Kerala. Out of the total 83 dairy cows presented with clinical mastitis, 22 *S. aureus* isolates could be obtained through bacteriological culture, morphological examination and biochemical tests. Antibiogram of the 22 *S. aureus* isolates using Kirby Bauer disk diffusion assay revealed that all the isolates were resistant to penicillin G (100 per cent). Thirteen isolates each (59.1 per cent) were found to be resistant to amoxicillin-sulbactam, cotrimoxazole and gentamicin each. Twelve isolates (54.55 per cent) were resistant to tetracycline followed by ten isolates (45.45 per cent) being resistant to ceftriaxone sulbactam and methicillin respectively. Majority of the isolates were found to be sensitive to Ceftriaxone, with only four (18.18 per cent) among the 22 isolates being resistant. The alarming levels of resistance exhibited by the *S. aureus* against different classes of antibiotics is of great concern since they might lead to serious economic and public health consequences and may serve as a reservoir for resistance and virulence genes.

Keywords: Antimicrobial resistance, Kirby Bauer disk diffusion assay, Mastitis, *Staphylococcus aureus*

For millennia, milk and dairy products has been considered as the nature's most complete food, and it continues to play a vital part in the diets of nearly 6 billion people worldwide. Milk and dairy products are a critical source of nourishment for these individuals whereas dairying acts as source of livelihood for dairy farmers, dairy processors, shop keepers, and stake holders in the dairy value chain.With the introduction of globalisation and liberal trade policies, the demand for safe and wholesome milk has risen to the top of global health agenda due to impending food security concerns. In this scenario, apart from the negative economic impact to the livelihoods of people, bovine mastitis poses a public health threat due to its potential for the transmission of many milk borne zoonotic diseases, antibiotic residues, bacterial toxins as well as organisms carrying numerous resistance and virulence factors.

Staphylococcus aureus is recognised as one of the primary contagious mastitis pathogen and a successful multi-host bacterium, which is consistently being identified from bovine mastitic milk. It causes severe economic loss due to both clinical and subclinical mastitis. Antimicrobials are extensively used in dairy industry to treat and prevent mastitis. However, the unscrupulous and imprudent therapeutic and prophylactic use of antimicrobials is known to instigate a drug induced selection pressure that results in the evolution of multi drug resistance. The emergence of antimicrobial resistance in *S. aureus* are gaining great importance in recent times due to its ability to resist therapy and expand into new host species following host switching events and subsequent adaptation through acquisition and/or loss of mobile genetic elements as well as further host-specific mutations (Richardson *et al*., 2018).

Hence, understanding the dynamics of acquisition of antibiotic resistance in *S. aureus* by a coordinated multidisciplinary surveillance system is of paramount importance in the effective eradication of this zoonotically significant pathogen. The Kirby Bauer disk diffusion assay is a low cost, easily adaptable tool that can be employed to assess the phenotypic resistance of an organism to various antibiotics.Therefore, the present study was conducted to evaluate the phenotypic antimicrobial susceptibility profile of *S. aureus* isolated from bovine mastitis which could help the clinicians in formulating strategies to achieve optimum containment of bovine mastitis and

*Corresponding author: krupaputhuparampil@gmail.com

the emerging antimicrobial resistance crisis.

Materials and Methods

Sample collection and study Area

A total of eighty-three dairy cows of varying age, parity and stages of lactation presented with clinical mastitis from various parts of Thrissur district, Kerala were examined, during the period from March 2019 to July 2020.

Approximately 5-10 ml of milk samples were collected aseptically from each animal and transferred to the laboratory, while maintaining the cold chain and is subjected to bacterial isolation and identification.

Bacteriological isolation and identification

Milk samples were thawed, mixed properly, and a loop full of milk sample was then cultured on to mannitol salt agar (MSA-M118, Himedia) and incubated for 24-48 hours at 37°C. The presumptive identification of *S. aureus* was done through colony characteristics on mannitol salt agar, Gram's staining and biochemical characterisation using oxidase, catalase and coagulase tests (Barrow and Feltham, 1993; Quinn *et al*., 2013). All the staphylococcal isolates obtained were further characterised up to species level with the help of a biochemical test kit procured from Himedia (HiStaph test kit, KB004) as per manufacturer's guidelines.

Antibiogram of Staphylococcus aureus

The phenotypic antimicrobial susceptibility profiling of the S. *aureus* isolates were performed by i*n vitro* Kirby Bauer disc diffusion assay (Bauer *et al*., 1966) by measuring the diameter of zone of inhibition (Fig. 1) as per the Clinical and Laboratory Standards Institute guidelines (CLSI, 2017).

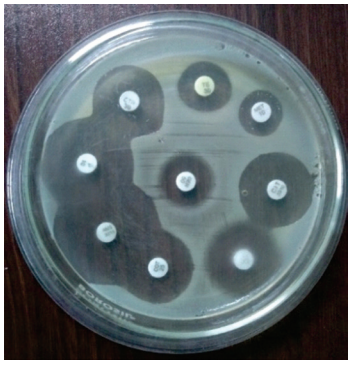

Fig. 1: Antimicrobial susceptibility testing of *S. aureus* isolates by disc diffusion assay

Preparation of the inoculum

Pure cultures were used as inoculum. Three to four similar colonies from 18- 24 h old pure cultures were picked up using a sterile nichrome loop and transferred into three millilitres of BHI broth (BHI broth; M210) and incubated at 37°C for four to six hours till light to moderate turbidity was developed.

Test procedure

Mueller-Hinton agar plates (MHA; M173, Himedia, India) were used to study the antibiotic sensitivity pattern of the isolates. For this, dehydrated culture media used for the study were reconstituted in distilled water as per manufacturer's instructions and sterilised by autoclaving at 121°C and 15 psi for 15 min. The sterilised molten medium was cooled to 45-50°C and poured in to sterile dry petri plates on a levelled surface, to a depth of 4 ± 0.2mm, allowed to solidify and then incubated at 37°C for 24 h to check the sterility.

A sterile non-toxic cotton swab on a wooden applicator was dipped into the standardized inoculum and pressed firmly against the upper inside wall of the tube to remove excess fluid in the swab. The entire agar surface of the plate was streaked with the swab three times, turning the plate at 60^{0} angle between the streaking and the inoculum was allowed to dry for 5-10 min with the lid in place.

Antibiotic discs with known concentration in microgram (mcg) or international unit (IU) per disc were used in the study. The details of the antibiotic discs are shown in the table below (Table 1).

Table 1. Antibiotic concentration of discs used for antibiogram (CLSI, standards)

Sl. No	Antibiotic disc used	Abbreviation and antibiotic concentration per disc
1.	Amoxicillin- sulbactam	AMS (30/15µg)
2.	Ceftriaxone	CTR (30µg)
3.	Ceftriaxone- Sulbactam	CIS (30/15µg)
4.	Cotrimoxazole	COT (23.75/1.25µg)
5.	Enrofloxacin	EX (10µg)
6.	Gentamicin	GEN (50 µg)
7.	Methicillin	MET (10µg)
8.	Penicillin G	P (10 U)
9.	Tetracycline	TE (30µg)

The antibiotic discs were applied under aseptic conditions with a distance of at least 24 mm apart. The plates were incubated immediately at 37°C and examined after 14-18 h or later if necessary. The clear zones were measured and the diameter of the zone of inhibition was recorded. Zone diameter values were measured inclusive of the disc diameter of six millimetre and compared with the standard inhibition zone chart provided by the manufacturer to find out sensitive and resistant antibiotics (CLSI, 2017).

Multiple Antibiotic resistance Index

Subsequent to the analysis of antibiogram, isolates showing phenotypic resistance to a minimum of the three different classes of antibiotics were considered as multidrug resistant *Staphylococcus aureus* (MDSA). Multiple Antibiotic Resistance (MAR) index is a tool to find out the spread of antibiotic resistant bacteria in a given population. The MAR index values for each *S. aureus* isolate and each antibiotic was calculated using the following formulas (Krumperman 1983):

$$\text{MAR index for antibiotics} = \frac{\text{Number of antibiotics to which an isolate was resistant}}{\text{Total number of antibiotics tested}}$$

Results and Discussion

The most common signs of clinical mastitis diagnosed by physical examination during the study period were swelling, redness, pain, heat, and abnormal colour and consistency of the mammary gland secretions followed by signs of systemic disturbances such as depression, anorexia, weakness, fever and enlargement of supramammary lymph nodes.

Bacterial isolation and identification

In the present study, isolation of bacteria from bovine mastitic milk was achieved by standard microbiological culture techniques. Upon initial inoculation of 83 mastitic milk samples on to MSA, 32 samples (38.55 per cent) did not produce any colonies whereas, 51 samples yielded bacterial growth (61.45 per cent). The bacterial isolates were identified based on their morphology and colony characteristics on selective media. The species level characterisation was done with the help of a biochemical test kit procured from Himedia (HiStaph test kit, KB004) as per manufacturer's guidelines and 22 isolates were identified as *S. aureus*. Thus, the overall isolation rate of *Staphylococcus aureus* from quarter-level mastitic milk samples were 26.5 per cent.The findings of the present study corroborate with Birhanu *et al*. (2017), Persson *et al*. (2011) and Kulaste *et al*. (2020) who observed a higher prevalence of Gram positive isolates like staphylococci and streptococci compared with coliforms. However, Perez *et al*. (2015) reported that 95.2 per cent of the isolates were Gram negative, implying a higher prevalence of environmental pathogens rather than contagious pathogens. These results are rather reasonable due to the variation in the agro-climatic condition, management practices and the pathogen ecology in the herd environment (Perez *et al*., 2015).

Antibiogram of S. aureus

The multifactorial aetiology of intramammary infection and its therapy refractory nature warrants the determination of the specific aetiological agent and its antimicrobial susceptibility profile before the initiation of therapeutic procedures. The disc diffusion assay is a simple, low-cost, easily interpretable qualitative typing method for the phenotypic profiling of AMR (Balouiri *et al*., 2016). It guides the clinicians in choosing the initial empirical treatment based on the resistance phenotype of the aetiological agent (Balouiri *et al*., 2016).

The result of the present study reports an alarming level of resistance among the *S. aureus* isolates towards Penicillin G, with all the isolates (100 per cent) being resistant to Penicillin (Table 2). Hence, it was found to be the least effective molecule against *S. aureus* whereas, ceftriaxone was found to be the most effective with only four isolates (18.18 per cent) being resistant. Thirteen isolates (59.1 per cent) each were found to be resistant against amoxicillin-sulbactam, sulphadiazine-trimethoprim and gentamicin. Twelve isolates (54.54 per cent) were resistant to tetracycline followed by ten isolates (45.45 per cent) each being resistant to ceftriaxone sulbactam and methicillin. The per cent of phenotypic resistance of *S. aureus* against Penicillin G (100 per cent) detected in our study was similar to reports from Amrithapriya (2019) and Khakpoor *et al.* (2011) but higher than that reported by Dorneles *et al.* (2019) (78.9 per cent) and Kulangara *et al*. (2017). However, the occurrence of 18.18 percent resistance to ceftriaxone and 54.54 per cent resistance to tetracycline in this study was much lower than the 62 per cent and 85 per cent resistance to the respective drugs, as reported by Dorneles *et al*. (2019) and Kulangara *et al*. (2017). In this study,

45.45 per cent of the isolates turned out to be resistant to methicillin. This is quite contrary to Amrithapriya (2019) who reported 94 per cent of the *S. aureus* isolates to be phenotypically resistant to methicillin. The 59.1 per cent resistance to sulphadiazine trimethoprim is almost in agreement with that of Dorneles *et al*. (2019) who reported 62 per cent of the isolates being resistant to the same. In the present study, the *in vitro* antibiogram of 22 *S. aureus* isolates against nine different antibiotics revealed that their resistance is increasing drastically with majority (68.18 per cent) of the isolates being multi drug resistant (MDR) and none of the isolates were susceptible to all the antibiotics studied. Raj (2018) and Amrithapriya (2019) also made a similar observation of MDR among *S. aureus* isolates from the nasal cavity of healthy pigs and bovine mastitic milk samples, respectively. However, Feng *et al*. (2016) performed *in vitro* antimicrobial resistance profiling of *S. aureus* isolated from bovine mastitis in North west China identified using nine different antimicrobial agents and found that all the isolates showed resistance towards at least one antimicrobial agent and 9.09 percent of the isolates were identified as MDR.

Staphylococcus aureus generally possess multidrug resistant genotype showing resistance to beta lactams, aminoglycosides fluroquinolones and macrolides because of the unique ability of the organism to respond quickly to each new antibiotic resulting in the development of resistance mechanisms (Kaur and Chate, 2015). The MAR index of greater than 0.2 suggests the presence of high risk source of contamination where antibiotics are commonly used, while MAR values of less than or equal to 0.2 are assumed to have originated for strains in which the antibiotics are rarely used (Krumperman, 1983).

Some of the potential reasons for the increased resistance towards certain antimicrobials might be the prolonged and imprudent use of that antimicrobial agent for treatment, as well as prophylaxis of mastitis in the study area. Increased availability of over the counter drugs and wide spread therapeutic and prophylactic use of intramammary and intramuscular broadspectrum antibiotics without professional guidance or bacteriological examination of milk samples is a common practice under field conditions in Kerala. The culling policy may be different, and the owners of smaller farms may keep cows, possibly chronically infected ones in the herd for a longer period of time. These conditions may exacerbate the antimicrobial resistance problem. Therefore, it could be inferred that judicious drug selection, careful treatment and detailed documentation in relation to bacteriological analysis of milk and antimicrobial susceptibility testing needs to be enforced in order to achieve optimum containment of bovine mastitis and AMR crisis in field settings.

Moreover, it is obvious from the study that the antibiogram of one region cannot be generalised to another region. This was in consonance with Singh *et al*. (2015) who concluded that the antibiogram may vary owing to the variations in the herd response, type of organism involved, location of infected sites, degree of udder induration, pharmacodynamics and pharmacokinetic behaviour of antibiotics in udder and milk, site of injection, antimicrobial sensitivity of udder pathogens, pH of milk and inflammatory exudates/barrier at the site. Another disadvantage of the phenotypic antibacterial resistance profiling was that the outcomes are reported on a qualitative basis and it cannot differentiate between the bactericidal or bacteriostatic effect and hence, subtle changes in susceptibility might not be evident (Balouiri

Table 2. Antibiogram and MAR index of *Staphylococcus aureus*

Sl. No	Antibiotic	Sensitive	Resistant	MAR Index
1.	Amoxicillin Sulbactam	9	13	0.066
2.	Ceftriaxone	18	4	0.020
3.	Ceftriaxone- sulbactam	12	10	0.050
4.	Cotrimoxazole	9	13	0.066
5.	Enrofloxacin	17	5	0.025
6.	Gentamicin	9	13	0.066
7.	Methicillin	12	10	0.050
8.	Penicillin G	0	22	0.1
9.	Tetracycline	10	12	0.060

(S: Sensitive, R: Resistant)

et al., 2016). Thus, it can be concluded that the results of antimicrobial susceptibility testing have to be combined with clinical information and experience while selecting the appropriate antibiotic. Above all, the dairy farmers should be made aware of the potential hazards associated with the indiscriminate use of antimicrobials.

Conclusions

The results of the present study revealed a high prevalence of *S. aureus* mastitis and an alarming level of resistance among the isolates from animals in the study area. This might be a serious threat to animal welfare as well as human health due to the possibility of direct transmission of staphylococci or its AMR determinants to human strains.Therefore, an integrated multidisciplinary surveillance strategy such as a 'One health approach' is an absolute necessity to report the occurrence of AMR as well as to formulate policy decisions in curbing the menace of AMR. Moreover, the study highlights the need for regular screening and *in vitro* testing, along with genomic and transcriptomic studies. This could enhance the knowledge about the local epidemiology of AMR in different strains of bacteria and is important for predicting the trends in resistance, detection of outbreaks, tracking the spread of infection, epidemiological surveillance, and control of infection.

Ethical approval and consent statement

The animal owners were informed about the purpose of the study and the verbal consent of each animal owner was obtained prior to the physical examination of cows and the collection of milk samples

Conflicts of interest

There were no conflicts of interest reported by the author (s).

Acknowledgement

The authors are thankful to the Kerala Veterinary and Animal Sciences University for providing the facilities needed for carrying out the research.

References

Amrithapriya M. G. 2019. Clinico-therapeutic studies of bovine mastitis caused by Staphylococcus aureus and its molecular typing. MVSc thesis, Kerala Veterinary and Animal Sciences University, Pookode, 154p.

Balouiri, M., Sadiki, M. and Ibnsouda, S. K., 2016. Methods for *in vitro* evaluating antimicrobial activity: A review. *J. Pharm. Anal*.:**6**: 71-79.

Barrow, C. I. and Feltham, R. K. A. 1993. Cowan and Steel's Manual for the Identification of Medical Bacteria. (3rd Ed.). Cambridge University Press, Great Britain, 331p.

Bauer, A. W., Kirby, M. M., Serris, J. S. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol*.:**45**: 493-96.

Birhanu, M., Leta, S., Mamo, G. and Tesfaye, S. 2017. Prevalence of bovine subclinical mastitis and isolation of its major causes in Bishoftu Town, Ethiopia. *BMC Res. Notes.*:**10**: 764-67.

Clinical and Laboratory Standards Institute. 2017. Performance standards for Antimicrobial Susceptibility testing (25th Ed.). Clinical and Laboratory Standards Institute, Wayne,15p.

Dorneles, E. M., Fonseca, M. D., Abreu, J. A., Lage, A. P., Brito, M. A., Pereira, C. R., Brandão, H. M., Guimarães, A. S. and Heinemann, M. B. 2019. Genetic diversity and antimicrobial resistance in *Staphylococcus aureus* and coagulase-negative Staphylococcus isolates from bovine mastitis in Minas Gerais, Brazil. *Microbiol. Open*.:**8**: p.e00736.

Feng, Y. A. N. G., Qi, W. A. N. G., Wang, X. R., Ling, W. A. N. G., Li, X. P., Luo, J. Y. Zhang, S. D. and Li, H. S. 2016. Genetic characterization of antimicrobial resistance in *Staphylococcus aureus* isolated from bovine mastitis cases in Northwest China. *J. Integr. Agric*.:**15**: .2842-47.

Kaur, D. C. and Chate, S. S. 2015. Study of antibiotic resistance pattern in methicillin resistant *Staphylococcus aureus* with special reference to newer antibiotic. *J. Glob. Infect. Dis.*:**7**: 9-10.

Khakpoor M., Safarmashaei, S. and Jafray, R. 2011. Study of milk extraxted from cows related to *Staphylococcus aureus* by culturing and PCR. *Global Veterinaria*.:**7:** 572-75.

Kulangara, V., Nair, N., Sivasailam, A., Sasidharan, S., Kollannur, J. D. and Syam, R. 2017. Genotypic and phenotypic β-lactam resistance and presence of PVL gene in Staphylococci from dry bovine udder. *PloS one*.:**12**: p.e0187277.

Kulaste, T.R., Chandra, M., Kaur, G., Narang, D., Gupta, D.K. and Arora, A.K. 2020. Prevalence of microorganisms causing mastitis in dairy animals and their antibiotic resistance. *Indian J. Vet. Med.*:**40**: 35-38.

Krumperman, P. H. 1983. Multiple antibiotic resistance indexing of E. coli to identify high-risk sources of faecal contamination of foods. *Appl. Envir. Microbiol*. **46**: 165-70.

Magiorakos, A.P., Srinivasan, A., Carey, R.T., Carmeli, Y., Falagas, M.T., Giske, C.T., Harbarth, S., Hindler, J.T., Kahlmeter, G., Olsson-Liljequist, B. and Paterson, D. T., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect*.:**18**: 268- 81.

Persson, Y., Nyman, A. K. J. and Grönlund-Andersson, U. 2011. Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. *Acta Vet. Scandinavica*.: **53** :36- 39.

Perez, S., De Meulemeester, L., de Kruif, A., Opsomer, G., Barkema, H. W. and De Vliegher, S. 2015. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. *J. Dairy Res*.:**74**: 474-.78.

Quinn, P., Markey, B., Carter, M. and Carter, G. R. 2013. In: Clinical Veterinary Microbiology. (2nd Ed) Mosby, St. Louis, 514p.

Raj, S. S. 2018. Occurrence and characterization of methicillin resistant Staphylococcus aureus in pigs. M.V.Sc thesis, Kerala Veterinary and Animal Sciences University, Pookode, 85p.

Richardson, E. J., Bacigalupe, R., Harrison, E. M., Weinert, L. A., Lycett, S., Vrieling, M., Robb, K., Hoskisson, P. A., Holden, M. T., Feil, E. J. and Paterson, G. K. 2018. Gene exchange drives the ecological success of a multi-host bacterial pathogen. *Nat. Ecol. Evol*.: **2**: 1468-78

Singh, K.P., Singh, R.V., Singh, P., Singh, S.K. and Singh, J.P. 2015. Comparative evaluation of antimicrobials for therapeutic management of bovine mastitis. *Intas Polivet*.:16:261-64.

Received : 18.01.2022
Accepted : 29.05.2022