Antimicrobial Resistance and Detection of Methicillin Resistance In Staphylococcus Species Isolated from Mastitis Cattle

Kislay Kumar1, Purushottam Kaushik1*, Anjay1, Archana1, Bhoomika1 and Pankaj Kumar2

Abstract

The study was aimed to isolate staphylococci from cattle and to determine their antibiotic resistance pattern. A total of 200 milk samples of cattle suffering from subclinical / clinical mastitis were included in the present study. Out of 200 samples processed for isolation, 173 isolates were confirmed as genus staphylococci based on PCR amplification of tstaG gene. All the confirmed isolates were further analyzed for antibiotic sensitivity for different antibiotics. The antibiogram of isolates in the present study revealed that maximum numbers of isolates have the resistance against oxacillin (62.42%) followed by cefoxitin (53.76%), Rifampicin (47.40%), erythromycin (38.15%) and gentamicin (31.79%). The study shows low resistance against clindamicin (29.48%), ciprofloxacin (13.29%) and minimum resistance in the staphylococci were observed against teicoplanin (7.32%) and tetracycline (1.73%). Methicillin resistance by PCR targeting mecA gene was detected among 38.73% of isolates, however multidrug resistance (MDR) were recorded in 53.76% isolates showing resistance to more than two groups of antibiotics.

Key words: Staphylococcus, mecA, antibiotic resistance, MDR.

Introduction

India has vast livestock resources, claiming highest milk producing country in the world, which play an important role in the economy of India. Livestock sector contributes 4.11% to the country GDP and 25.6% of total Agriculture GDP of India. Mastitis in dairy animals has a significant impact on quality milk production and animal health as well (Le Maréchal et al., 2011). Staphylococci has been recognized as the most frequent bacterial pathogen of bovine mastitis in most of the countries including India (Gillespie et al., 2009; Nazneen et al., 2014; Brahma et al., 2022) Staphylococcus has been considered as the true mastitis pathogens virulence factors by most of the researcher worldwide (Hoque et al., 2018; Hosseinzadeh and Saei., 2014) with high level of antimicrobial resistance. In India 50% of milking animals are reported to be suffering with mastitis (Dua 2001) leading to an estimated annual loss of INR.7165 crores (Bansal and Gupta 2010). In modern dairy herds, subclinical mastitis is the major form of mastitis, affecting more than 20 to 50% of cows (Wilson et al., 1997; Pitkala et al., 2004) which remains latent and poses more serious economic concern to the dairy sector as its incidence is much higher in dairy herd (Shaheen et al., 2016). The indiscriminate uses of antibiotics for treatment of infected animals result in emergence of resistance to antibiotics in micro-organisms (Philips et al., 2004; Levy, 1982). Emergence of antibiotic resistance in staphylococci (both S. aureus and Coagulase Negative staphylococci) associated with subclinical and clinical mastitis in animals has increased significantly worldwide (Jain et al., 2004; Hoque et al. 2018; Phophi et al., 2019; Brahma et al., 2022). The present study was aimed to determine the occurrence of staphylococcus in mastitic animals of Bihar and to the antibiotic resistance pattern of isolates.

Material and methods

Isolation and identification of staphylococci

A total of 200 milk samples from cattle with subclinical (137) and clinical (63) form of mastitis were collected aseptically from four district viz. Gaya, Patna, Vaishali & Sitamarhi of Bihar. All the samples were transported to the department of Veterinary Public Health & Epidemiology laboratory within 12h of collection under refrigerated condition for Isolation and identification of Staphylococcus species. The samples were enriched in Brain Heart Infusion broth at 37 C for 24 h. (Palilu et al., 2017). Then, a loopful of inoculum from enrichment broth with turbidity was streaked on mannitol salt agar plate followed by incubation at 37 C for 24 h and examined for the growth of Staphylococcus. Both mannitol fermenter and non-fermenter colony from mannitol salt agar plate was picked and examined under oil immersion after Gram's staining as per the method described by Agarwal et al., (2003). Presumptive colonies with characteristic morphology further confirmed by catalase test and PCR amplification of tstaG gene for confirmation of isolates as staphylococci. The primers sequence used for amplification of tstaG gene are indicated in Table-1. The template DNA

✉ drkaushikvet@gmail.com

1. Department of Veterinary Public Health & Epidemiology, BVC, BASU Patna
2. Department of Veterinary Microbiology, BVC, BASU Patna

used for PCR was prepared by boiling and snaps chilling from isolates (Kaushik et al., 2014). The PCR reaction was prepared in 25 µl volume each containing 2.5 µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM MgCl2), 0.5 µl of dNTP mixture (10 mM each), 2 µl (10 pmol/µl) each of forward and reverse primers, 1µl (1 unit) Taq DNA polymerase, 5 µl of bacterial lysate and 12 µl nuclease free water. The cycling conditions were optimized at an initial denaturation phase of 94oC for 5 min, followed by 40 amplification cycles of denaturation at 95oC for 30 sec, annealing at 55oC for 30 sec and elongation at 72oC for 30 sec, with a final elongation phase at 72oC for 10 min.

Antibiotic susceptibility and detection of mecA gene

All the confirmed Staphylococcus isolates under study were examined to determine the antibiotic resistance pattern by disc diffusion method (Bauer et al., 1966; Mahato et. al. 2017) using 9 antibiotic discs (Hi-Media, Mumbai) viz. Oxacillin (1µg), Ciprofloxacin (5µg), Teicoplanin (30µg), Cefoxitin (30µg), Erythromycin (15µg), Rifampicin (5µg), Clindamycin (2µg), Gentamicin (10µg) and Tetracycline (30µg). The isolates found resistant to oxacillin were screened for the presence of mecA gene by PCR. The primer used for detection of mecA is indicated in Table-1. The PCR reaction was prepared in 25 µl volume each containing 2.5 µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM MgCl2), 0.5 µl of dNTP mixture (10 mM each), 2 µl (10 pmol/µl) each of forward and reverse primers, 1µl (1 unit) Taq DNA polymerase, 5 µl of bacterial lysate and 12 µl nuclease free water. The cycling conditions were optimized at an initial denaturation phase of 94oC for 5 min, followed by 32 cycles of denaturation at 95oC for 60 sec, annealing at 55oC for 60 sec and elongation at 72oC for 60 sec, with a final elongation phase at 72oC for 10 min

Results and discussion:

A total of 173 staphylococci were isolated and confirmed using genus specific PCR targeting tstaG gene amplification (Fig-1), from the milk samples of mastitic cattle. The confirmed isolates screened for antibiotic susceptibility revealed different resistance pattern for isolates. The resistance profile of isolates shows maximum resistance against oxacillin (62.42%), cefoxitin (53.76%), rifampicin (47.40%) and erythromycin (38.15%) whereas isolates have low resistance against gentamicin (31.79%) and clindamycin (29.48) and minimum resistance was found against ciprofloxacin (13.29%), teicoplanin (7.32%) and tetracycline (1.73%). The results of the present study corroborate with the findings of Mahto et al., (2017) wherein oxacillin and cefoxitin were reported as most resistant drugs in staphylococci isolated from milk of mastitic animals. The low resistance of gentamicin reported in the present study is accordance to the previous report by Kumar et al., (2011) and Akindolire et al., (2015). The high susceptibility of isolates to teicoplanin corroborates with the findings of Sahebekhtiari et al., 2011; Juliano et al., 2022; Brahma et al., 2022. The minimum resistance to tetracycline in staphylococcal isolates from mastitic animals may be useful in the treatment of mastitis in animals. The analysis of antibiogram of the isolates revealed the occurrence of MDR in 53.76% isolates of staphylococci

The occurrence of methicillin-resistant Staphylococci in bovine mastitis is a growing challenge (Paji et al., 2014; Turutoglu et al., 2006). PCR based detection of mecA gene (fig-2) for methicillin resistance in the study revealed that 38.73% (67/173) isolates were positive for the mecA gene and also showing phenotypic resistance against oxacillin. The present finding agrees to the finding of Guimarães et al., (2017); Hoque et al., (2018); Algammal et al., (2020) which reports the occurrence of high percentage of methicillin resistant mecA positive staphylococci in milk from animals with mastitis. Though the 38.73 % isolates were positive for mecA gene, 33.50% (67/200) animals with subclinical or clinical mastitis have infection with methicillin resistant staphylococci. Out of the mecA positive isolates in the present study, 44.44% (28/63) and 43.33% (39/90) isolates were from clinical and subclinical mastitis milk, respectively. The occurrence of high percentage of mecA positive isolates in subclinical mastitis indicates trends of high antimicrobial resistance among lactating animals and a major public health threat. The analysis of antibiotic resistance pattern of isolates for MDR staphylococci revealed 53.76% (93/173) of isolates as MDR showing resistance against more than two groups of antibiotics, of which 33.33% (31/93) of isolates were from clinical mastitis and 66.66% (62/93) of isolates were from subclinical mastitis cases. The study again reveals the presence of more MDR staphylococci in subclinical cases of mastitis agreeing to the findings of Hoque et al., (2018).

Conclusion:

The study concludes that the staphylococci are the major bacteria involved in mastitis (clinical or subclinical) in lactating cattle. The occurrence of high percentage of antibiotic resistant staphylococcus against various antibiotic such as oxacillin, cefoxitin, rifampicin and gentamicin may pose serious challenge to use of antibiotics in mastitis treatment. The occurrence of MDR staphylococci in mastitis, predominantly in subclinical cases of mastitis act as a potential threat to both human and animal health.

Acknowledgement:

The Authors acknowledge the contribution of Dean, Bihar Veterinary College and Director Research, Bihar Animal Sciences University Patna for providing necessary facilities and support to carry out the present work. We are thankful to the Honb'le vice chancellor, BASU for extending financial support to the study.

Table.1. Sequence of the primer for PCR amplification specific to tstaG and mecA genes.

Sl. No.	Primer Sequences (5'- 3')	Target gene	Product size (bp)	Reference
1.	F: GGCCGTGTTGAACGTGGTCAAATCA	*tstaG*	370bp	Morot-Bizot et al., 2004
	R: TACCATTTCAGTACCTTCTGGTAA			
2.	F: GTAGAAATGACTGAACGTCCGATAA	*mecA*	310bp	Braoios *et al*., 2009
	R: CCAATTCCACATTGTTTCGGTCTAA			

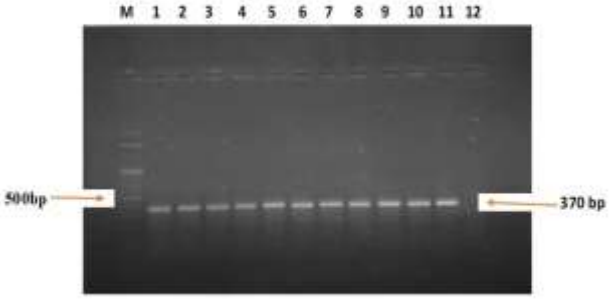

Fig. 1 PCR amplification of *tstaG* gene
M: 100bp DNA ladder
L12: Negative control

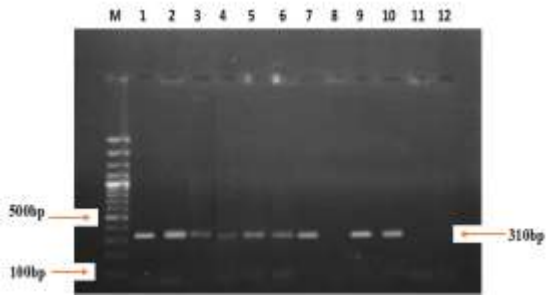

Fig. 2 PCR amplification of *mecA* gene
M: 100bp DNA ladder
L12: Negative control

References

Agarwal, R. K., Bhilegaonkar, K. N., Singh, D. K., Kumar, A. and Rasthore, R. S. (2003). Laboratory manual for the isolation and identification of food borne pathogens. 1st Edition. IVRI, Izzatnagar. 2003: 38-9.

Akindolire, M. A., Babalola, O. O. and Ateba, C. N. (2015). Detection of antibiotic resistant Staphylococcus aureus from milk: A public health implication. International journal of environmental research and public health. 12(9): 10254-10275.

Algammal, A.M., Enany, M.E., El-Tarabili, R.M., Ghobashy, M.O.I. and Helmy, Y.A. (2020). Prevalence, Antimicrobial Resistance Profiles, Virulence and Enterotoxins-Determinant Genes of MRSA Isolated from Subclinical Bovine Mastitis in Egypt. Pathogens. 9(5):362.

Bansal, B. K. and Gupta, D. K. (2010). Economic analysis of bovine mastitis in India at Punjab. Indian Journal of Dairy Science. 62: 337-345.

Bauer, A. W., Kirby, W. M. M., Sheris, J. and Truck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. 145: 225-230.

Brahma, U., Suresh, A., Murthy, S., Bhandari, V. and Sharma, P. (2022). Antibiotic Resistance and Molecular Profiling of the Clinical Isolates of Staphylococcus aureus Causing Bovine Mastitis from India. Microorganisms. 10(4):833.

Braoios, A., Fluminhan Junior, A., Pizzolitto, A. C. (2009). Multiplex PCR use for Staphylococcus aureus identification and oxacillin and mupirocin resistance evaluation. Journal of Basic and Applied Pharmaceutical Sciences. 30(3):303-307.

Dua, K. (2001). Incidence etiology and estimated loss due to mastitis in India. Indian dairyman. 53: 41-48.

Gillespie, B.E., Headrick, S.I., Boonyayatra, S. and Oliver S.P. (2009). Prevalence and persistence of coagulase-negative Staphylococcus species in three dairy research herds. Veterinary Microbiology. 134:65–72.

Guimarães, F.F., Manzi, M.P., Joaquim, S.F., Richini-Pereira, V.B. and Langoni H. (2017). Short communication: Outbreak of methicillin-resistant Staphylococcus aureus (MRSA)-associated mastitis in a closed dairy herd. Journal of Dairy Science. 100(1):726-730.

Hoque, M.N., Das, Z.C., Rahman, A.N.M.A., Haider, M.G. and Islam M.A. (2018). Molecular characterization of Staphylococcus aureus strains in bovine mastitis milk in Bangladesh. International Journal of Veterinary Science & Medicine. 6(1):53-60.

Hosseinzadeh, S. and Saei, D.H. (2014). Staphylococcal species associated with bovine mastitis in the North West of Iran: Emerging of coagulase-negative Staphylococci. International Journal of Veterinary Science & Medicine. 2:27–34.

Jain, A., Agarwal, J. and Bansal S. (2004). Prevalence of methicillin-resistant, coagulase- negative staphylococci in neonatal intensive care units: findings from a tertiary care hospital in India. Journal of Medical Microbiology.53:941–944.

Juliano, L.C.B., Gouvêa, F.L.R., Latosinski, G.S., Fabri, F.H.H., Salvador, T.B., Guimaraes, F.F., Ribeiro, M.G., Langoni, H., Rall, V.L.M., Hernandes, R.T., Leite, D.S. and Pantoja J.C.F. (2022). Species diversity and antimicrobial resistance patterns of Enterococcus spp. isolated from mastitis cases, milking machine and the environment of dairy cows. Letters in Applied Microbiology. 75(4):924-932.

Kaushik, P., Anjay, Kumari, S., Bharti, S. K. and Dayal, S. (2014). Isolation and prevalence of Salmonella from chicken meat and cattle milk collected from local markets of Patna, India. Veterinary World. 7(2): 62-65.

Kumar, R., Yadav, B. R. and Singh, R. S. (2011). Genetic determinants of antibiotic resistance in Staphylococcus aureus isolates from milk of mastitic crossbred cattle. Current Microbiology. 60: 379–386.

Le Maréchal, C., Seyffert, N., Jardin, J., Hernandez, D., Janm, G., Rault, L. (2011). Molecular basis of virulence in Staphylococcus aureus mastitis. PLoS ONE. 6:e27354.

Levy, M. (1982). The Lazarsfeld-Stanton Program Analyzer: An Historical Note. Journal of Communication. 32(4):30 – 38.

Mahato, S., Mistry, H. U., Chakraborty, S., Sharma, P., Saravanan, R. and Bhandari, V. (2017). Identification of Variable Traits among the Methicillin Resistant and Sensitive Coagulase Negative Staphylococci in Milk Samples from Mastitic Cows in India. Frontiers in Microbiology. 8: 1446.

Maniati, M., Petinaki, E., Maniatis, A.N., Spiliopoulou, I., Petropoulou-Mylona, D., Malamou-Lada, H., Spaliara, L. and Koutsia-Carouzou, C. (2005). Rapid increase in numbers of Staphylococcus epidermidis strains with reduced susceptibility to teicoplanin in Greece. International journal of antimicrobial agents, 25(4):346-348.

Morot-Bizot, S. C., Talon, R. and Leroy, S. (2004). Development of a multiplex PCR for the identification of Staphylococcus genus and four staphylococcal species isolated from food. Journal of Applied Microbiology. 97: 1087–1094.

Nazneen, N.I., Farzana, Z., Chowdhury, A.M.M.A., Mannan, A., Kamaruddin, K.M. and Siddiki A.M.A.M.Z. (2014). Characterization of bovine subclinical mastitis caused by Staphylococcus aureus in southern Bangladesh by bacteriological and molecular approaches. Asian Journal of Biological Science.7:1–12.

Paji , M.J., Raši , Z.B., Velebit, B.M., Boboš, S.F., Mihajlovi -Ukropina, M.M., Radinovi , M. ., Galfi, A.L., Petkovi , J.M. and Troja anec, S.I. (2014). The prevalence of methicillin resistance and Panton-Valentine leukocidin synthesis genes in Staphylococcus aureus isolates of bovine and human origin. Veterinarski achieve 84(3):205-214.

Palilu, P. T. and Budiarso, T. Y. (2017). Isolation and identification of Staphylococcus sp. in powdered infant milk. AIP Conference Proceedings.1844: 020016.

Phillips, S. J., Dudk, M., Schapire, R. E. (2004). A maximum entropy approach to species distribution modeling. In: Proceedings of the 21st International Conference on Machine Learning, ACM Press, New York. 655–662.

Phophi, L., Petzer, I.M., Qekwana, D.N. (2019). Antimicrobial resistance patterns and biofilm formation of coagulase-negative Staphylococcus species isolated from subclinical mastitis cow milk samples submitted to the Onderstepoort Milk Laboratory. BMC Veterinary Research. 15(1):420.

Pitkala, A., Haveri, M., Pyorala, S., Myllys, V. and Honkanen-Buzalski, T. (2004). Bovine mastitis in Finland 2001-Prevalence, distribution of bacteria, and antimicrobial resistance. Journal of Dairy Science.87(8): 2433-2441.

Sahebekhtiari N., Nochi Z., Eslampour M.A., Dabiri H., Bolfion M., Taherikalani M., Khoramian B., Zali M.R. and Emaneini M. (2011). Characterization of Staphylococcus aureus strains isolated from raw milk of bovine subclinical mastitis in Tehran and Mashhad. Acta Microbiologica et Immunologica Hungarica. 58(2):113-121.

Shaheen, M., Tantary, H. A. and Nabi, S. U. (2016). A Treatise on bovine mastitis: disease and disease economics, etiological basis, risk factors, impact on human health, therapeutic management, prevention and control strategy. Journal of Advances in Dairy Research. 4: 150.

Turutoglu, H. U. L. Y. A., Ercelik, S., and Ozturk, D. (2006). Antibiotic resistance of Staphylococcus aureus and coagulase-negative staphylococci isolated from bovine mastitis. Bulletin-Veterinary Institute In Pulawy. 50(1): 41.

Wilson, D. J., Gonzalez, R. N. and Das, H. H. (1997). Bovine mastitis pathogens in New York and Pennsylvania: prevalence and effects on somatic cell count and milk production. Journal of Dairy Science. 80(10): 2

*****