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Screening of Milk Borne *Staphylococcus aureus* for Resistance against Beta Lactam Antibiotics

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ABSTRACT

Background: A study was carried out to screen milk borne *Staphylococcus aureus* for resistance against Beta lactam antibiotics.

Methods: A total of 45 milk samples were collected over a period of three months from large animal outpatient unit of Madras Veterinary College Hospital, Chennai. Upon collection of samples, ABST followed by its growth in Mannitol Salt Agar was carried out as part of the phenotypic screening. Genotypic screening for *Staphylococcus* screening was done with the help of PCR by using *nuc* and *mec* A primers. MIC for ceftriaxone and cloxacillin was carried out with the samples that were found positive for *Staphylococcus aureus*. The antibiotic sensitivity pattern is presented: Fluoroquinolones (87.5% sensitive), aminoglycosides (72.5% sensitive), Amoxicillin-Clavulanic acid (Amoxyclave) (72.5% sensitive). The MSA positive samples were subjected to molecular identification with the help of PCR.

Result: The results revealed 10 samples positive for *Staphylococcus aureus* and 5 among them positive for mecA gene. The MIC results were as follows: MIC_{50}-10.95µg/ml and MIC_{90}- 87.510.95µg/ml for ceftriaxone and MIC_{50}- 43.75 µg/ml and MIC_{90}- 87.5µg/ml for cloxacillin, indicating emergence of resistance. However, further studies are required in a larger sample size that can help us to attain more conclusive results.

Key words: ABST, Ceftriaxone, Cloxacillin, MIC, PCR.

INTRODUCTION

Clinical and sub clinical mastitis are significantly reported diseases in dairy cows causing severe economic losses to dairy farmers by way of reduced milk yield and quality (Kumar *et al.* 2017; Zeryehun *et al.* 2017). In dairy cows, mastitis caused by *Staphylococcus aureus* is commonly subclinical, manifested by elevated concentrations of leucocytes (primarily neutrophils) in milk (elevated somatic cell counts, SCC) (Rainard *et al.,* 2017). Failure of treatment in mastitis is due to indiscriminate use of antibiotics without testing *in vitro* sensitivity (Awandkar *et al.,* 2013). There is a greater need to emphasize the importance of judicious usage of antibiotics to reduce the development of resistance posing massive challenge in the treatment of bacterial diseases. In view of the growing incidence of anti-microbial resistance in large animals, the present study has been undertaken to screen sensitivity pattern of commonly used antibiotics in mastitis treatment, followed by isolation, microbiological and molecular characterization of *Staphylococcus organisms.*

MATERIALS AND METHODS

Study area

The study was carried out in the Department of Veterinary Pharmacology and Toxicology and Department of Veterinary Public Health, Madras Veterinary College, Tamil Nadu University of Veterinary and Animal Sciences, Chennei- 7, India.

Antibiotic sensitivity testing

A total of 45 milk samples were collected from mastitis affected cows and screened for the presence of *Staphylococcus* organisms. Antibiotic sensitivity pattern was carried out by disc diffusion method on Mueller-Hinton agar plates (Balouiri *et al.,* 2016).

Isolation of *Staphylococcus* organisms

The organisms in the milk sample were subjected to growth on Mannitol Salt Agar. Each isolate was cultured on Mannitol Salt Agar and incubated at 37°C for 24 hours. The procedure used in the preparation of Mannitol Salt Agar is standardized (Shields and Yang, 2006).

Minimum inhibitory concentration

MICs of Ceftriaxone and Cloxacillin against *Staphylococcus aureus* were determined using broth dilution method. The method described by Andrews, (2001) was followed by Wiegand *et al.* (2008).

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Polymerase chain reaction

PCR amplification of *nuc* gene was carried out for molecular characterization of *Staphylococcus aureus*. Detection of *mec* A in the presumptive isolates was done using PCR (Brakstad *et al.,* 1992; Pournajaf *et al.,* 2014).The primer sequences for *mec*A gene and *nuc* gene and their cyclic temperatures for amplification are given in Table 1 and Table 2 respectively.

RESULTS AND DISCUSSION

Antibiotic sensitivity testing

Results indicated a sensitivity pattern of antibiotics in the following order: Levofloxacin (87.5%), Ciprofloxacin (82.5%), Co-trimoxazole (77.5%), Amoxyclav (72.5%), Gentamicin (72.5%), Amikacin (67.5%), Tetracycline (42.5%), Cephalothin (42.5 %), Cefuroxime (37.5%), Azithromicin (25%) Ceftriaxone (72.5%), Cloxacillin (67.5%). Verma *et al.,* (2018) conducted a study to see the antibiotic sensitivity pattern in mastitis affected cows and results indicate 65.96% and 63.83% sensitivity for Ceftriaxone and Amoxicillin, respectively. The results of Antibiotic Sensitivity testing are depicted in Table 3 and Fig 1.

Isolation of *Staphylococcus aureus*

Among the total number of milk samples collected, 88.88% of the samples were confirmed to be *Staphylococcus* organisms by virtue of growth of yellow coloured colonies on MSA plates. The colonies were aseptically picked and transferred to 50% glycerol stock solution for the purpose of storage. Mannitol Salt Agar (MSA) has been used since 1945 as a selective medium for the isolation of pathogenic *Staphylococci* (Blair *et al.,* 1967; Chapman, 1945). So, it possibly indicates that some of the samples that were found positive for *Staphylococcus* organism belonged to genus other than *Staphylococcus aureus* (Sheilds and Tsang, 2006). The growth of yellow coloured colonies is depicted in Fig 2.

Polymerase chain reaction

Among the positive isolates of *Staphylococcus* organisms that were subjected to *nuc* gene amplification, 25% of the isolates were confirmed to be positive for *Staphylococcus aureus* as indicated by PCR results. The positive *Staphylococcus* organisms were also subjected to amplification of *mec*A gene using PCR and results revealed the presence of *mec* A gene in 12.5% of Staphylococcus isolates that were subjected to amplification of the concerned gene. Amplification of *mec*A gene was done with the help of PCR using *mec* A primer. The agarose gel electrophoresis of *mec A* and *nuc* gene PCR products are depicted in Fig 3.

Minimum inhibitory concentration

MIC_{50} and MIC_{90} for ceftriaxone against *Staphylococcus aureus* were recorded to be 10.95 µg/ml and 87.5 µg/ml, respectively. Similarly, MIC_{50} and MIC_{90} of Cloxacillin were found to be 43.75 µg/mland 87.5 µg/ml, respectively against *Staphylococcus aureus*. Current FDA Ceftriaxone breakpoints are <4 µg/ml (susceptible), 8 µg/ml (intermediate) and >16 µg/ml (resistant). The FDA Cloxacillin breakpoints are < 2µg/ml (Susceptible) and > 4µg/ml (Resistant). The MIC study for Cloxacillin was found to be

Table 1: Primer Sequence for *mec* A and *nuc* gene.

Gene	Sequence (5′-3′)	Amplicon Size
Mec A	(F) AAA ATC GAT GGT AAA GGT TGG C	532 bp
	(R) AGT TCT GCA GTA CCG GAT TTG C	
Nuc	(F) GTGCTGGCATATGTATCGCAAATTGT	181 bp
	(R) TACGCCCTAATCTGTTTGTGATGC	

Table 2: PCR Cyclic conditions for amplification of *nuc* and *mec* A gene.

Name of gene	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
Nuc	94°C/5m	94°C/30s	54°C/30s	72°C/30s	72°C/10m
mec	94°C/5m	94°C/1 m	55°C/1 min	72°C/2 m	72°C/5m

Table 3: Antibiotic sensitivity testing.

Antibiotic	Sensitive	Moderately sensitive	Non-sensitive
Gentamicin	29	12	1
Ciprofloxacin	33	8	1
Azithromycin	10	20	12
Tetracycline	17	12	13
Co-trimoxazole	31	4	8
Amikacin	27	11	4
Levofloxacin	35	6	1
Amoxyclav	29	6	7
Cefuroxime	15	14	13
Cephalothin	17	7	18
Ceftriaxone	29	7	6
Cloxacillin	27	10	5

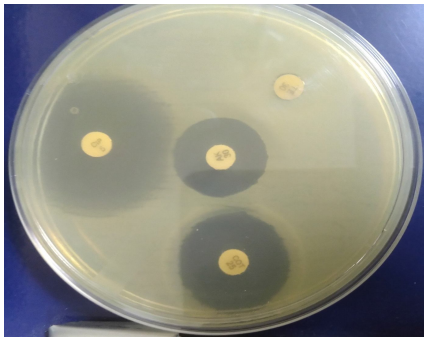

Fig 1: Antibiotic sensitivity test.

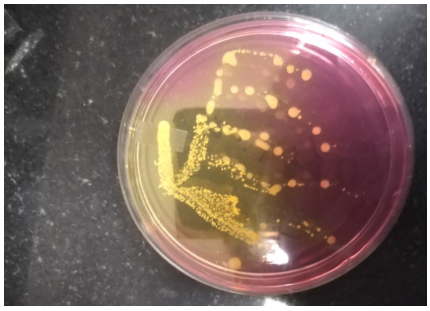

Fig 2: Mannitol Salt Agar plates showing the growth of *Staphylococcus* organism.

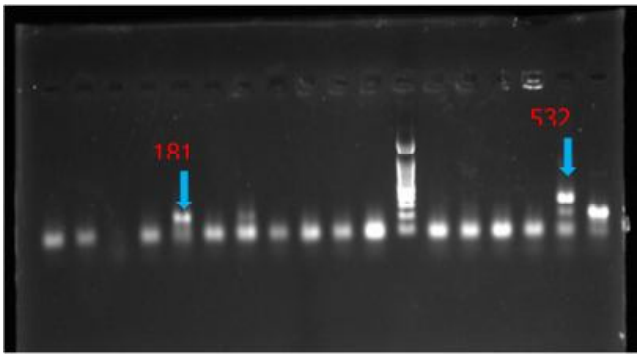

Fig 3: Polymerase chain reactions showing the positive for *nuc* and *mec* A.

43.75 µg/ml - 87.5 µg/ml against *Staphylococcus aureus*. The results of the present study suggest that the isolates of *S. aureus* were resistant to Ceftriaxone which would involve a mechanism of PBP mutation. MIC values indicate resistance of *Staphylococcus aureus* to both ceftriaxone and cloxacillin. However, study on a greater number of isolates is required to characterize the extent and type of resistance.

CONCLUSION

The MIC values clearly indicate that *Staphylococcus aureus* is resistant to both Ceftriaxone and Cloxacillin. There is a heightened need to be judicious in our choice of antibiotics and also work out the right combination of antibiotics to stem the development of resistance. Also, further studies are required involving larger geographical area to arrive at a better conclusion.

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REFERENCES

Andrews, J.M. (2001). Determination of minimum inhibitory concentrations. The Journal of Antimicrobial Chemotherapy. 48: 5-16.

Awandkar, S.P., Bhikane, A.U. and Kulkarni, M.B. (2013). Antibiotic resistance trends in clinical bovine mastitis. Biolife. 1: 139-143

Balouiri, M., Sadiki, M. and Ibnsouda, S.K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis. 6: 71-79.

Blair, E.B., Emerson, J.S. and Tull, A.H. (1967). A new medium, salt mannitol plasma agar, for the isolation of *Staphylococcus aureus*. American. Journal of Clinical Pathology. 47: 30-39.

Brakstad, Odd, G., Kjetill, Aasbakk. and Johan, A., Maeland. (1992) Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. Journal of Clinical Microbiology. 30: 1654-1660.

Chapman, G.H. (1945). The significance of sodium chloride in studies of Staphylococci. J. Bacterio. 50: 201-203.

Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: 20th Informational Supplement. Wayne, PA: CLSI; 2010.

Kumar, N., Manimaran, A., Sivaram, M., Kumaresan, A., Jayekumar, S., Sreela, S., Moovethan, P. and Rajendran, D. (2017). Influence of clinical mastitis and its treatment outcome on reproductive performance in crossbred cows: A retrospective study. Veterinary World. 10: 485- 492.

Pournajaf, A., Abdollah, A., Leyla, G., Mahmoud, K., Tahmineh, N. and Hassan, A. (2014). PCR-based identification of methicillin– resistant *Staphylococcus aureus* strains and their antibiotic resistance profiles. Asian Pacific Journal of Tropical Biomedicine. 4: S293-S297.

Rainard, P., Foucras, G., Fitzgerald, J.R., Watts, J.L. and Middleton J.R. (2017). Knowledge gaps and research priorities in *Staphylococcus aureus* mastitis control. Transboundary and Emerging Diseases. 65: 149-165.

Shields, P. and Tsang, A.Y. (2006). Mannitol salt agar plates protocols. Available from the MicrobeLibrary website: http://www. microbelibrary. org/library/laboratory-test/3034-mannitol-salt-agar-plates-protocols.

Verma, H., Rawat, S., Sharma, N., Jaiswal, V. and Singh R. (2018). Prevalence, bacterial etiology antibiotic susceptibility pattern of bovine mastitis in meerut. Journal of Entomology and Zoological Studies. 6: 706-709.

Wiegand, I., Hilpert, K. and Hancock, R.E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols. 3: 163.

Zeryehun, T. and Abera, G. (2017). Prevalence and bacteriological isolates of mastitis in Dairy farms in selected districts of Eastern Harrarghe Zone, Eastern Ethiopia. Journal of Veterinary Medicine. Article ID 6498618.