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

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10.18805/ag.D-5123

**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 outpatients 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 aureus* screening was done with the help of PCR by using nuc and mecA 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, Chennai- 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).

**How to cite this article:** Anurag, B., Ramasamy, T., Ramesh, S., Sriraam, K.S., Kalaiselvi, L., Deepak, S.J., Kannan, R.G. and Arunaman, C.S. (2021). Screening of Milk Borne *Staphylococcus aureus* for Resistance against Beta Lactam Antibiotics. Agricultural Science Digest. 41(1): 113-115. DOI: 10.18805/ag.D-5123.

**Submitted:** 02-01-2020  **Accepted:** 09-07-2020  **Online:** 07-01-2021
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 mecA 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 Staphyloccoci (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 mecA 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 mecA 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

MIC₅₀ and MIC₉₀ for ceftriaxone against Staphylococcus aureus were recorded to be 10.95 µg/ml and 87.5 µg/ml, respectively. Similarly, MIC₅₀ and MIC₉₀ of Cloxacillin were found to be 43.75 µg/ml and 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             |
3.75 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.

**ACKNOWLEDGEMENT**

Authors are thankful to the Professor and Head, Department of Clinics, Madras Veterinary College Teaching Hospital, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India for providing the facilities required to conduct this study.

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