Original Research

PCR (*nuc* gene) Based Identification of Antibiotic Resistant *Staphylococcus aureus* Isolated from Mastitis

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Rec. Date:	May 06, 2019 08:04
Accept Date:	Sep 19, 2019 18:32
DOI	10.5455/ijlr.20190506080452

Abstract

Milk sample from cattle showing clinical signs of mastitis were collected and subjected to cultural characterization and biochemical analysis to identify the etiology. Gram staining of the isolated organism was identified as Staphylococcus spp by biochemical tests. The isolate was confirmed as Staphylococcus aureus in Mannitol salt agar which produced yellow colored colonies and assessed for its susceptibility to antibiotics by disc diffusion method of antibiotic sensitivity test. The isolate showed sensitivity to Enroflxacin, Tetracycline and resistance to Amoxicillin, Ceftriaxone, Penicillin G, Cotrimoxazole and Streptomycin. The resistant isolates were screened for the presence of nuc gene in Staphylococcus aureus for methicillin resistance by polymerase chain reaction. The polymerase chain reaction amplified 270bp amplicon indicating the presence of nuc gene in Staphylococcus aureus isolated from the milk sample. The presence of methicillin resistant Staphylococcus aureus in the milk sample emphasizes the occurrence and role of milk in the transfer of antimicrobial resistance between animals and human beings.

Key words: Antibiotics, Mastitis, Methicillin Resistant *Staphylococcus aureus* (MRSA), *nuc* gene, Polymerase Chain Reaction (PCR), Resistance, *Staphylococcus aureus*

How to cite: Venkatachalam, S., Kalaivanan, R., & Kuppanan, S. (2019). PCR (nuc gene) Based Identification of Antibiotic Resistant *Staphylococcus aureus* Isolated from Mastitis. International Journal of Livestock Research, 9(11), 176-182. doi: 10.5455/ijlr.20190506080452

Introduction

Mastitis in cattle is a most common disease of dairy animals with severe economic and public health impact. The disease is caused by range of etiological agents including gram-positive and gram-negative bacteria, mycoplasmas, fungi and algae. Transmission is primarily through contaminated hands or materials, milking machine and fomites. The severity of the inflammation can be classified into sub-clinical, clinical and chronic forms, and its degree is dependent on the nature of the causative pathogen and on the age, breed, immunological health and lactation state of the animal (Yalcin, 2000).

Staphylococcus aureus (S. aureus), a normal inhabitant of udder of dairy animals is the most frequent cause of mastitis (Katsuda *et al.,* 2005; Hosseinzadeh and Saei, 2014). Intramammary infections (IMIs) caused by this bacterium are highly transmittable, especially during milking (Sears and McCarthy, 2003; Olde Riekerink *et al.,* 2008). This pathogen is an increasingly recognized and most frequently isolated etiology of bovine mastitis in most countries (Gillespie *et al.,* 2009). Antimicrobials in different combinations are being used to treat the condition but in vain. The first strain of MRSA was reported in the UK and Europe in 1961, just less than 1 year after the introduction of methicillin for clinical use. Since that time, MRSA has become a major public health problem worldwide and the prevalence of MRSA has been dramatically rising in recent years (Louie *et al.,* 2002). More than 90% *S. aureus* strains are resistant to penicillin (Livermore, 2004). Thus, rapid and exact methods for identification of MRSA in clinical specimens are essential for accurate diagnosis and antimicrobial therapy (Huletsky *et al.,* 2004).

This bacterium also suppresses phagocytosis and cell mediated immunity and produces an enzyme that inactivates most penicillin-based treatments (De Oliveira *et al.,* 2000). The organisms have evolved many mechanisms like antimicrobial resistance to persist in the environment. One such mechanism is the production of an extracellular thermostable nuclease (TN*ase*). It is an endonuclease, degrading both DNA and RNA and the enzymatic activity can resist 100°C for at least 1 h (Lachica *et al.,* 1972). The TN*ase* protein has been well characterized and the gene coding for it viz., the *nuc* gene is used in many laboratories for the identification of methicillin resistant *S. aureus* isolate. The present study aims to identify the presence of *nuc* gene in the *S. aureus* isolated from the milk sample of dairy cow affected by mastitis and correlate the resistance to penicillin and amoxicillin antibiotics used in field conditions.

Materials and Methods

Sample Collection

Milk sample from the right fore quarter and right hind quarter of *Gir* cattle with swelling of udder and teat, altered consistency of milk along with drop in production was collected aseptically in a sterile container and transported to the laboratory.

Identification of the Organism

Primary isolation of organism from the collected milk sample was carried out in the nutrient agar plate and incubated at 37˚C for 24 hours to observe colony characteristics. Thin smear was prepared from the bacterial colony grown in the nutrient agar plate in a grease free glass slide and was subjected to gram staining. Biochemical test such as catalase, coagulase and oxidase test was also performed for identification (Chakraborthy *et al.,* 2011). After identifying the isolate, a colony from the nutrient agar was streaked in selective media, mannitol salt agar (MSA) and incubated at 37˚C for 24 hours.

DOI 10.5455/ijlr.20190506080452

Antibiotic Sensitivity Test

The *S. aureus* isolated from milk sample was subjected to antimicrobial sensitivity test by disc diffusion method following CLSI guidelines with commonly used antibiotics *viz.*, enroflxacin (10 µg), tetracycline (30 µg), amoxicillin (30 µg), ceftriaxone (30 µg) penicillin G (10 units), cotrimoxazole (10 µg), streptomycin (10 µg). Zone of inhibition was measured in diameters and studied using norms of the National Committee on Clinical Laboratory Standards (NCCLS). The isolates resistant to penicillin G and amoxicillin was subjected to screen for the presence of *nuc* gene coding for thermo stable endonuclease by PCR.

DNA Extraction by Hot Cold Lysis

The protocol described by Liu *et al.*, 2001 was followed for extraction of DNA. *S. aureus* isolate was inoculated in nutrient broth and kept overnight at 37˚C in the incubator. The culture was centrifuged at 10,000 rpm for 3 min. The supernatant was discarded and pellet was used for extraction of nucleic acids. Pellet was washed with PBS and resuspended in 20 µl nuclease free water and boiled for 5-10 min and then kept on ice. After boiling and cooling, the lysate was centrifuged at 10,000 rpm for 2 min to remove debris. The supernatant containing DNA was collected and stored at -20 ºC.

Quantification of DNA by Nanodrop

The DNA content in the supernatant collected from the culture extracted by hot cold lysis method was measured using Nanodrop instrument. One microliter of nuclease free water was placed initially as blank. Subsequently, 1µl of DNA extracted from the sample was placed in the Nanodrop and concentration of DNA was determined. The quantity of DNA was calculated by spectrophotometric method. The optical density (OD) at 260 and 280 nm was taken in Nanodrop with distilled water as reference. Purity of DNA was estimated on the basis of OD ratio at 260:280 nm. The DNA content of the samples with acceptable purity (*i.e.*, ratio between 1.6-1.8) was quantified using the following formula and used for PCR.

Concentration of DNA (µg/ml) = OD at 260 x dilution factor x 50

where, 50 is concentration of *ds*DNA expressed in µg/ml at OD of one.

Primers Used for Amplification of *nuc* gene of *S. aureus*

The isolates were tested by using PCR analysis to amplify the part of the *nuc* gene, encoding thermostable nuclease, specific for *S. aureus* (Brakstad *et al.*, 1992).

Forward	5'- GCGATTGATGGTGATACGGTT-3'	Expected amplicon size- 270 bp
Reverse	5'- AGCCAAGCCTTGACGAACTAAAGC-3'	

 DOI 10.5455/ijlr.20190506080452

Polymerase Chain Reaction

The PCR was carried out using 200µl capacity thin wall PCR tubes with a final volume of 20µl. The reaction mixture is as follows-

S. No.	Components	Quantity (µl)
1	Taq master mix red – (2X) (Ampliqon, USA)	10
2	Forward primers (10 pmol/ µl)	1
3	Reverse primers (10 pmol/ µl)	1
4	Template DNA	1
5	Nuclease free water	7
Total Volume		**20**

The PCR tubes containing the mixture were tapped gently and spun briefly. The PCR tubes with all the components were transferred to thermal cycler (Multigene Optimax, Labnet, USA). No template control (NTC) was also used in each PCR without template DNA. The PCR cycle conditions recommended by Brakstad *et al.* (1992) were followed as mentioned below-

Steps and Conditions of Thermal Cycling for *nuc* gene PCR

S. No.	Steps	Temperature	Time
Step 1	Initial denaturation	95ºC	5 min
Step 2	Denaturation	94ºC	60 sec
Step 3	Annealing	55ºC	30 sec
Step 4	Extension	72ºC	90 sec
Step 5	Step 2-4 for 35 cycles		
Step 6	Final extension	72ºC	4 min
Step 7	Holding temperature	4ºC	Hold

Results and Discussion

Mastitis is an economically important contagious disease producing severe impact on the dairy animals and the farmers. Though, panel of etiologies have been described to cause bovine mastitis, Schukken *et al.*, 2009, reported that 3 percent of all animals are infected with *S. aureus* and represents 10 to 12 percent of all clinical mastitis infections (Tenhagen *et al.*, 2009). Further the threat posed by the etiological agent in the guise of antimicrobial resistance is an alarming concern since the pathogen is communal pathogen of humans and animal. Though, various laboratory techniques are existing customizing the rapid and accurate techniques for identification of these resistant bacteria can contribute to the earlier diagnosis and timely treatment of infections.

In the present study, the milk sample was collected from the cattle with the clinical signs of hot and painful udder producing watery milk with flakes. Cultural examination revealed that the colonies were large, circular, smooth, shiny and appeared golden yellow in color which was in concurrence with Ochei and Kolhatkar, 2008, who reported Staphylococci colonies on most media are round, smooth, raised and

DOI 10.5455/ijlr.20190506080452

measuring 1 – 2mm in diameter. The gram staining of the smear prepared from the colonies grown on nutrient agar revealed gram positive cocci arranged in clusters (Fig.1).

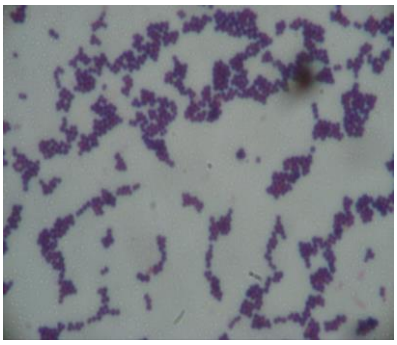

Fig. 1: Gram stained smear showing gram positive cocci arranged in clusters

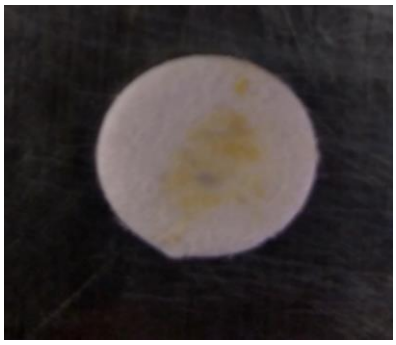

Fig. 2: Oxidase disc showing no color change on addition of culture indicating negative reaction

Fig. 3: An effervescence of oxygen gas produced within few seconds indicating catalase positive.

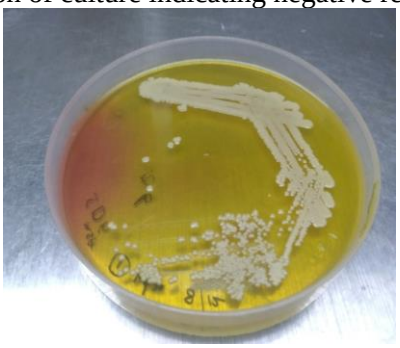

Fig. 4: Mannitol Salt agar- Colonies showing fermentation of mannitol indicative of *S. aureus*

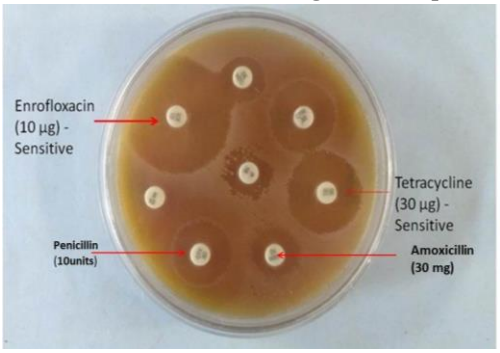

Fig. 5: Antimicrobial sensitivity test of *S.aureus* isolate showing susceptibility and resistance to different antibiotics

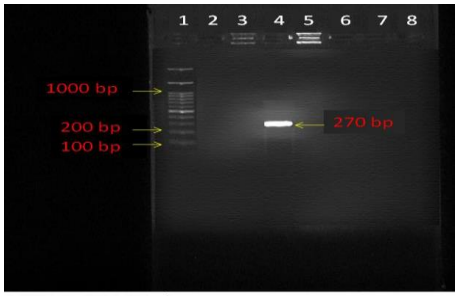

Fig. 6: PCR amplification of *nuc* gene(270 bp)

Biochemical characterization of the isolate revealed catalase positive, coagulase positive and oxidase negative reactions (Fig. 2 &3) indicative of presence of *S. aureus* in the milk sample. The isolate inoculated in mannitol salt agar appeared yellow in color specifying fermentation of the sugar and production of acids confirming the presence of *S. aureus* (Fig. 4). According to Bautista-Trujillo *et al.*, 2013, rapid isolation and identification of the *S. aureus* pathogen is a major goal of diagnostic microbiology. A variety of selective and /or differential culture media have been used to isolate and identify the organism (Thaker *et*

DOI 10.5455/ijlr.20190506080452

al., 2013). The use of culture media for *S. aureus* isolation in combination with coagulase activity as secondary tests has improved the accuracy of identification (Bautista-Trujillo *et al.*, 2013). The isolates subjected to antimicrobial sensitivity test to determine the sensitivity pattern against commonly used antibiotics for treatment were found to be sensitive to enroflxacin (10 µg), tetracycline (30 µg) and resistant to amoxicillin (30 µg), ceftriaxone (30 µg) penicillin G (10 units), cotrimoxazole (10 µg) and streptomycin (10 µg)(Fig. 5).

The concentration of DNA extracted by hot cold lysis method from the culture of the isolate was quantified to contain 1880.5 ng/µl in nano drop. Brakstad *et al.*, 1992 reported that the *nuc* PCR detected < 20 viable *Staphylococcus aureus* cells or correspondingly low levels (0.69pg) of extracted DNA in saline. In this study, the PCR could amplify 270 bp *nuc* gene from the isolate confirmed the presence of methicillin resistance gene responsible for the resistance (Fig. 6). The results of the sensitivity test could be positively correlated to the PCR indicating the occurrence of antibiotic resistance. As per Thomson *et al.*, 1999 selective pressure favors the evolution of resistant strains as a result of misuse and over use of antibiotics. In the present study, the prolonged use of penicillin group of antibiotics in the recent years for the treatment of mastitis and other illnesses might have induced the resistant strains.

According David *et al.*, 2010, identification of *S. aureus* using PCR amplification of the *nuc* gene (~270 bp) is considered as a gold standard method. Moreover, Sahebnasagh *et al*., 2014, the *nuc* gene is more specific in detecting isolates belonging to the species *S. aureus* compared to the 16S rRNA gene that is common to the genus *Staphylococcus* and will detect other *Staphylococcus* species in addition to *S. aureus.* Similarly, in the present study, PCR using nuc gene primers has successfully amplified the 270 bp product indicating the existence of penicillin resistance in the isolates of mastitis. Hence, *nuc* gene PCR based identification and determination of antimicrobial resistance is an effective and rapid means to identify and control the spread of resistance.

Conclusion

In conclusion, the present study confirms the existence of penicillin resistant *Staphylococcus aureus* and its role in causing mastitis among dairy cattle. Isolation and identification along with molecular methods can be safely relied for the diagnosis of antibiotic resistant strains of organisms.

References

1. Bautista – Trujillo GU, JL Solorio – Rivera, I Renteria – Solórzano, SI Carranza – Germán, JA Bustos – Martinez, RI Arteaga – Garibay, VM Baizabal – Aguirre, M Cajero – Juárez, A Bravo – Patiño, and JJ Valdez – Alarcón. 2013. Performance of culture media for the isolation and identification of *Staphylococcus aureus* from bovine mastitis. *J. Med. Microbiol.*, 62 (pt 3): 369 – 376. doi.10. 1099/jmm.0. 046284 – 0.
2. Brakstad, O.G., Aasbakk, K. and Maeland, J.A., 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *J. Clin. Microbiol.*, 30(7):1654-1660.

 DOI 10.5455/ijlr.20190506080452

3. Chakraborty SP, Mahapatra SK and Roy S. 2011. Biochemical characters and antibiotic susceptibility of *Staphylococcus aureus* isolates. *Asian Pacific Journal of Tropical Biomedicine*, 1 (3): 212 – 216.
4. David P. K., Cyrus, N., Fred, A. K., Alfred O, Moses, S. and Ann N. 2010. Identification of *Staphylococcus aureus*: DN*ase* and mannitol salt agar improve the efficiency of the tube coagulase test. *Ann. Clin. Microbiol. Antimicrob.*; 9: 23.
5. De Oliveira AP, Watts JL, Salmon SA and Aarestrup FM. 2000. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and United States. *J Dairy Sci;* 83:855–62.
6. Gillespie BE, Headrick SI, Boonyayatra S and Oliver SP. 2009. Prevalence and persistence of coagulase-negative *Staphylococcus* species in three dairy research herds. *Vet Microb;* 134:65–72.
7. Hosseinzadeh S and Saei DH. 2014. Staphylococcal species associated with bovine mastitis in the North West of Iran: Emerging of coagulase-negative *Staphylococci*. *Int J Vet Sci Med;* 2:27–34.
8. Huletsky A, Giroux R, Rossbach V, Gagnon M and Vaillancourt M. 2004. New Real-Time PCR Assay for rapid detection of methicillin-resistant *Staphylococcus aureus* directly from specimens containing a mixture of Staphylococci. *J Clin Microb*; 42(5):1875–1884.
9. Katsuda K, Hata E, Kobayashi H, Kohmoto M, Kawashima K and Tsunemitsu H. 2005. Molecular typing of *Staphylococcus aureus* isolated from bovine mastitic milk on the basis of toxin genes and coagulase gene polymorphisms. *Vet Microbiol;* 105:301–5.
10. Lachica, R. V. F., P. D. Hoeprich, and H. P. Riemann. 1972. Tolerance of *Staphylococcal thermonuclease* to stress. *Appl. Microbiol*. 23:994-997.
11. Livermore DM. The need for new antibiotics. 2004. *Clin Microbiol Infect*.; 10:1–9.
12. Louie L, Goodfellow J, Mathieu P, Glatt A and Louie M. 2002. Rapid detection of methicillin-resistant staphylococci from blood culture bottles by using a multiplex PCR assay. *J Clin Microb*; 40(8): 2786–2790.
13. Olde Riekerink R.G.M., H. W. Barkema, D. F. Kelton and D. T. Scholl. 2008. Incidence rate of clinical mastitis on Canadian dairy farms. *J. Dairy Sci;* 91:1366-1377.
14. Ochei J and Kolhatkar. 2008. Medical Laboratory Science: theory and practice, 7th reprint 2008, Tata McGraw – Hill Publishing Co. Ltd. P 644 – 674.
15. Sahebnasagh R, Saderi H and Owlia P. 2014. The prevalence of resistance to methicillin in Staphylococcus aureus strains isolated from patients by PCR method for detection of mecA and *nuc* genes. *Iran. J. Public Health*.43(1): 84–92.
16. Schukken, Y. H., R. N. Gonzalez, L. L. Tikofsky, H. F. Schulte, C. G. Santisteban, F. L. Welcome, G. J. Bennett, M. J. Zurakowski, and R. N. Zadoks. 2009. CNS mastitis: Nothing to worry about? *Vet. Microbiol.* 134(1-2): 9-14.
17. Sears P.M, and K.K. McCarthy. 2003. Management and treatment of staphylococcal mastitis. *Vet. Clin. North Am. Food Anim. Pract*; 19 ,171-185.
18. Tenhagen, B. A., I. Hansen, A. Reinecke, and W. Heuwieser. 2009. Prevalence of pathogens in milk samples of dairy cows with clinical mastitis and in heifers at first parturition. *Journal of Dairy Research* 76(2): 179-87.
19. Thaker HC., MN Brahmhatt, and JB Nayak .2013. Isolation and identification of Staphylococcus aureus from milk and milk products and their drug resistance patterns in Anand, Gujarat. *Vet. World;* 6(1): 10 – 13. doi. 10.5455/vetworld.2013. 10 – 13.
20. Thomson, K.S.C.C. Sanders, and E.S. Moland. 1999. Antimicrob. Agents Chemother.43:1393-1400.
21. Yalcin C, 2000. Cost of mastitis in Scottish dairy herds with low and high sub-clinical mastitis problems. *Turk J Vet Anim Sci*, 24: 465-472.

DOI 10.5455/ijlr.20190506080452