Agricultural Sciences Journal
Available online at http://asj.mnsuam.edu.pk/index.php
ISSN 2707-9716 Print
ISSN 2707-9724 Online
https://doi.org/10.56520/asj.v5i1.235

Research Article

ANTIBIOTIC RESISTANCE AND MOLECULAR DETECTION OF *nuc* AND *mecA* GENES OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM MASTITIC MILK

Narmeen Tariq[*1,2], Sajjad Ur Rahman[2], Muhammad Tariq Javed[3], Aftab Ahmad Anjum[1], Hafza Zahira Manzoor[4], Muhammad Hunain Ahmed[3], Aira Tariq[5], Sami Ullah Khan Bahadur[3]
[1]*Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.*
[2]*Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan.*
[3]*Department of Pathology, University of Agriculture, Faisalabad, Pakistan.*
[4]*Department of experimental and translational medicine, University of insubria, Varese, Italy.*
[5]*Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan.*
**Corresponding Email: tariqnarmeen333@gmail.com*

Abstract

The emergence of methicillin resistant *Staphylococcus aureus* (MRSA) is mainly due to the presence of resistant genes. The objective of this study is to perform antibiotic susceptibility profiling and molecular detection of *nuc* and *mecA* genes of *Staphylococcus aureus* isolated from mastitic milk. A total of 105 milk samples were collected from dairy farms in the Faisalabad region. They were cultured on Staph-110 media and mannitol salt agar. Biochemical tests included catalase and coagulase, were also performed for the identification of *Staphylococcus aureus*. Antibiotic susceptibility profiling of MRSA was done by disc diffusion method and molecular screening of *Staphylococcus aureus* for the detection of *nuc* and *mecA* genes was done by using PCR. Selected 8 antibiotics based on previous history being commonly used in dairy practices. The prevalence of *Staphylococcus aureus* observed by PCR was 58.09%. Oxacillin (1µg) and vancomycin (30µg) showed 100% resistance, while enrofloxacin (5µg), amoxicillin (25µg), ampicillin (10µg), oxytetracycline (1µg), gentamycin (10µg) and tylosin (30µg) showed 1.64, 55.74, 73.77, 13.11, 1.64 and 11.74% resistance, respectively. Susceptibility percentages of enrofloxacin (5µg), amoxicillin (25µg), ampicillin (10µg), oxytetracycline (1µg), gentamycin (10µg) and tylosin (30µg) were 27.87, 37.70, 14.75, 19.67, 98.36 and 9.84%, respectively. Enrofloxacin (5µg), amoxicillin (25µg), ampicillin (10µg), oxytetracycline (1µg) and tylosin (30µg) showed 70.49, 6.56, 11.47, 67.21 and 78.69% intermediate resistance against MRSA. Among the mastitic cases, 58.09% were positive for *Staphylococcus aureus*. These isolates were susceptible to gentamycin in 98.36% and tylosin in 78.69% cases, so these antibiotics can be used for the treatment of mastitis.

Keywords: Antibiotics, Resistance, Mastitis, Bovines, MRSA
(Received: 6, January 2023, Accepted: 21, March 2023) Cite as: Tariq. N., Rahman. S. U., Javed M. T., Anjum. A. A., Manzoor. H. Z., Ahmed. M. H.,Tariq. A., Bahadur. S. U. K., 2023 Antibiotic resistance and molecular detection of nuc and meca genes of *Staphylococcus aureus* isolated from mastitic milk. Agric. Sci. J. 5(1): 17-24.

1. INTRODUCTION

The Dairy sector is an important source of income, food, and nutritional security that also improves the well-being of smallholder dairy farmers in Pakistan. Mastitis is a major issue for this industry, causing production losses that include low milk production, increase in labor cost, and decreased milk quantity (Ali *et al*., 2011). *Staphylococcus aureus* is a common cause of bovine mastitis, resulting in both clinical and subclinical mastitis (Hoekstra *et al.*, 2020). Clinical mastitis is characterized by swelling of the udder, changes in milk appearance, increased body temperature and redness of the udder, while subclinical mastitis can only be distinguished by laboratory tests. Cows with subclinical mastitis are a source of infection to other cows in the herd. To improve animal health and to reduce economic losses, it is necessary to minimize mastitis prevalence by different measures. Mastitis has been mostly treated with long-acting antibiotics, due to acute inflammation of the udder

tissue. Poor farm management and injudicious use of antibiotics can augment Methicillin-resistant *Staphylococcus aureus* (MRSA) emergence in bovines (Joshi *et al.,* 2014). MRSA has been reported as a transmissible zoonotic disease. Bovine milk samples from Faisalabad District showed a 34% prevalence of MRSA, 30% from cattle and 38% from buffalo (Aqib *et al.*, 2017). Many MRSA isolates showed resistance to different antibiotics; oxacillin (100%), penicillin (100%), tetracycline (55.6%) and vancomycin (44.6%), while sulfamethoxazole and gentamicin recorded the least levels of 11.1 and 5.6%, respectively (Umaru *et al.,* 2013; Atiq *et al.*, 2021). Ciprofloxacin, moxifloxacin, linezolid, and trimethoprim/sulfamethoxazole drugs showed highest efficacy against MRSA (Aqib *et al.,* 2017). Methicillin resistance is caused by the production of penicillin-binding protein-2a (PBP-2a), having decreased binding affinity for β-lactams and is carried out by mecA gene. There are two regulatory genes on mec DNA; mec1 and mecR1, that controls the expression of mecA and they are present the upstream of mecA, encode signal transducer protein and the mecA repressor protein (Lee, 2003). However, nuc and mecA gene expression can be detected by multiplex RT-PCR assay. It detects thermostable nuclease encoding nuc genes and it is specific for *Staphylococcus aureus,* while mecA gene that encodes for PBP-2a, brings β-lactam antibiotic resistance (Costa *et al.,* 2005). Therefore, nuc and mecA gene were targeted for the detection of mastitis and to check the methicillin-resistant *Staphylococcus aureus* in milk samples. So the hypothesis of current study is that *Staphylococcus aureus* is the major organism involved in mastitis and is susceptible to various antibiotics, while the resistant strains have genes responsible for the same. That's why study was planned to find out the antibiotic susceptibility of *Staphylococcus aureus* and molecular screening of nuc and mecA genes of *Staphylococcus aureus.*

2. MATERIALS AND METHODS

2.1.Sample collection

A total of 105 number milk samples were randomly collected from different dairy animals of the Faisalabad region after confirming with a 3% surf field mastitis test (SFMT). History was taken from the owner about the duration of disease, age and previous treatment of animals to analyze the most used drugs in the treatment of mastitis to figure out the resistance of drugs used. These samples were then stored at 4℃ until they were processed in the laboratory.

2.2. Isolation, purification and identification of *Staphylococcus aureus*

The SFMT-positive samples were then streaked on Staph-110 media for culture isolation of the organm. The isolates were then cultured on mannitol salt agar (MSA) to isolate and purify the *Staphylococcus aureus*. The gram staining was also performed. Different biochemical tests were also used to identify the *Staphylococcus aureus* that included catalase, coagulase and rabbit plasma test (Kateete *et al*., 2010).

2.3.Molecular testing

For molecular detection, the *nuc* and *mecA* genes of *Staphylococcus aureus* isolated from mastitic milk samples (n=105) were targeted. The DNA was extracted from bacterial colonies colonies grown on agar using thermo-scientific GeneJET geneomic DNA Purification Kit (Lot# 00777285). Thermal cycler conditions for nuc and mecA gene were given as reported by Kalorey *et al.* (2007) and Stegger *et al.* (2017), respectively.

1.1.Antibiotic susceptibility testing

Muller Hinton agar was prepared to check the antibiotic susceptibility through disc diffusion method. Following antibiotic discs of OXOID company: enrofloxacin 5µg, vancomycin 30µg, oxacillin 1µg, tylosin 30µg, amoxicillin 25µg, ampicillin

Table 1: Primers used for the detection of nuc and MecA gene:

Targeted gene	Primers	Primer Sequence	Reference
Nuc	Nuc-F Nuc-R	CGATTGATGGTGATACGGTT ACGCAAGCCTTGACGAACTAAAGC	Kalorey *et al.*, 2007
MecA	MecA-F MecA-R	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	Stegger *et al.*, 2017

Table 2: Susceptibility, resistance and intermediate resistance percentage of 8 different antibiotics used against *Staphylococcus aureus.*

Antibiotics	**Susceptible**	**Resistance**	**Intermediate**
Oxacillin (1µg)	-	61 (100%)	-
Vancomycin (30µg)	-	61 (100%)	-
Enrofloxacin (5µg)	17 (27.87%)	1 (1.64%)	43 (70.49%)
Amoxicillin (25µg)	23 (37.70%)	34 (55.74%)	4 (6.56%)
Ampicillin (10µg)	9 (14.75%)	45 (73.77%)	7 (11.47%)
Oxytetracycline (1µg)	12 (19.67%)	8 (13.11%)	41 (67.21%)
Gentamycine (10µg)	60 (98.36%)	1 (1.64%)	-
Tylosine (30µg)	6 (9.84%)	7 (11.47%)	48 (78.69%)

10µg, gentamycin 10µg, oxytetracycline 1µg were picked and placed on plates. Kirby Bauer method was used. Standard suspension of each strain was spread. Discs were placed and plates were incubated at 37°C for 24 hours. For determination of strain against specific antibiotic, zone of inhibitions were observed and compared with CLSI standards.

3. RESULTS:

3.1. Samples collection:

A total of 105 samples were confirmed positive for mastitis with 3% SFMT from dairy farms of Faisalabad region. Clinical samples in cow and buffalo were 24 and 14, respectively, while subclinical samples were 40 and 27, respectively.

3.2. Isolation, purification and identification of *Staphylococcus aureus*:

On staph-110 media, 74/105 samples showed round, moderate and golden yellow colonies were observed (Figure 1A). The golden yellow colonies were also present on 61/105 samples cultured on mannitol salt agar media (Figure 1B). As a result of gram staining, 74/105 samples showed purple colour stained grapes like cocci under microscope at 100X.

As a result of the catalase test, all 105 samples showed bubble formation, when bacterial colonies were mixed with H_2O_2 (Figure 1D). Coagulation was also seen in

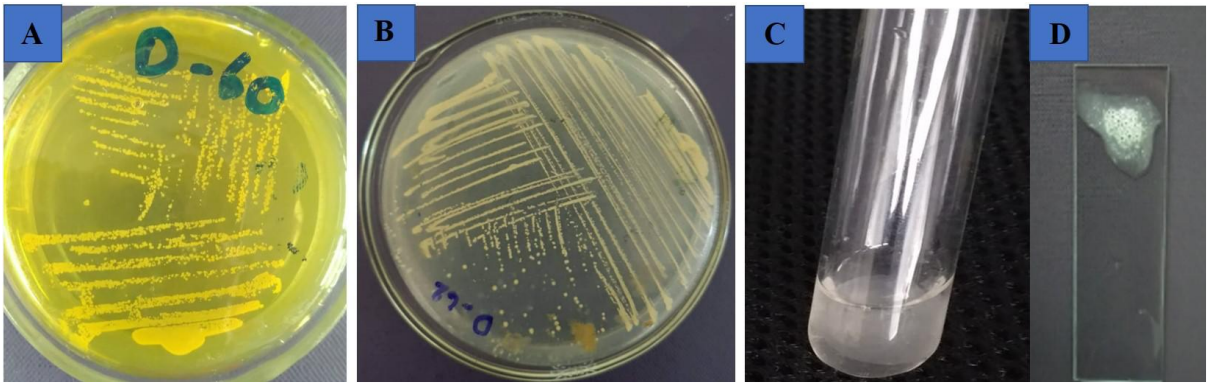

Figure 1 (A): Golden yellow colonies of *Staphylococcus aureus* on staph-110 media, (B) Golden yellow colonies of *Staphylococcus aureus* on MSA, (C) Coagulates test resulted into coagulation and (D) Catalase test resulted into bubble formation.

the test tubes of 61/105 samples as a result of coagulase test (Figure 1C).

3.3. Molecular detection:

Out of 105 samples, 61 of them were PCR positive to nuc and mecA gene, yielded product length of 279 and 162bp, respectively.

3.4. Antibiotic susceptibility profiling results:

Antibiograms were prepared against 8 commonly used antibiotics for the detection of Methicillin resistant *Staphylococcus aureus* that included enroflaxacin (5µg), vancomycin (30µg), oxacillin (1µg), tylosin (30µg), amoxacillin (25µg), ampcillin (10µg), gentamycine (10µg) and oxytetracycline (1µg). Out of 61 positive *Staphylococcus aureus* isolates, oxacillin (1µg) and vancomycin (30µg) showed 100% resistance (Figure 2). Enrofloxacin (5µg) showed 27.87% susceptibility, 1.64% resistance and 70.49% intermediate resistance. Amoxacillin (25µg) showed 37.70% susceptibility, 55.74% resistance and 6.56% intermediate resistance. Ampicillin (10µg) showed 14.75% susceptibility, 73.77% resistance and 11.47% intermediate resistance (Table 2). Oxytetracyclin (1µg) showed 19.67% susceptibility, 13.11% resistance and 67.21% intermediate resistance.

Gentamycine (10µg) showed 98.36% susceptibility and 1.64% resistance. Tylosine (30µg) showed 9.84%

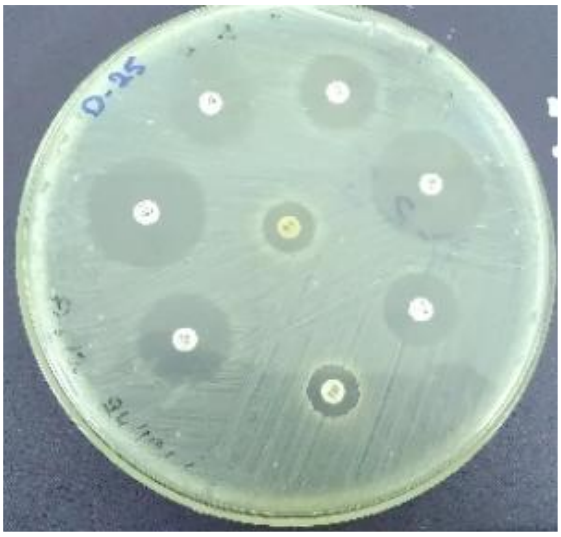

Figure 2: Antibiotic sensitivity test results showed resistant to most of the antibiotics.

susceptibility, 11.47% resistance and 78.69% intermediate resistance against MRSA (Table 2). After statistical analysis, it was found that chi-square and 95 percent confidence interval have to be worked out.

4. DISCUSSION

Mastitis is a major issue in dairy industry of Pakistan that causes direct and indirect losses (Ali *et al.*, 2011). The *Staphylococcus aureus* is one of major cause of chronic mastitis. Abo-Shama (2014) also reported that there were many pathogens identified in dairy industry, *Staphylococci* were the most important causative agents and some other agents like *Streptococci, Bacillus, Salmonellae, Pseudomonas, Corynebacterium, Escherichia and Klebsiella* species have

also been recognized that causes mastitis in bovines. *Staphylococcus aureus* produces a range of virulence factors and extracellular proteins as well as toxins (Akineden *et al.*, 2001). The MRSA has public health significance and veterinary concerns worldwide and one of the sources is mastitic milk (Umaru *et al.*, 2013). From different dairy farms of Faisalabad region, 105 milk samples were randomly collected for the detection of mastitis. The prevalence rate of *Staphylococcus aureus* found was 58% but in Pakistan previous reports indicated it to be 34.2% (Maalik *et al.*, 2019). The difference in percentages may have been due to samples in this study being collected from conventional dairy farms, where management of animals was very poor, resulting in higher prevalence of disease. The present study showed that proportion of sub-clinical mastitis was high, it can be due to many factors including animals were kept on barn land, poor management, lactation period and age.

On staph-110 media, 74/105 samples showed golden yellow colonies while 61/105 samples showed golden yellow colonies, when cultured on mannitol salt agar media. As a result of gram staining, 74/105 samples showed purple colored grapes like cocci at 100X under microscope. Kateete *et al.* (2010) also reported yellow colored colonies on mannitol salt agar after 24 hours of incubation at 37°C and Gram positive cocci in response to gram staining that indicated *Staphylococcus aureus*. The 74/105 (70.48%) isolates were positive to the catalase test as Mandel (1975) reported that all *Staphylococcal* strain showed positive reaction with hydrogen peroxide. While 61/105 (58.09%) isolates were positive for the coagulase test. This may be due to the use of two media for the isolation of *Staphylococcus aureus* from milk samples, i.e., Staph-110 and mannitol salt agar. As mannitol salt agar also supports the growth of *Staph epidermidis* and *Micrococcus* which are non-lactose fermenter and coagulase negative (Becker *et al.*, 2014). Sperber *et al.* (1975) also observed 439 (86%) strains positive for the coagulase test out of 508 for *Staphylococcus aureus*.

For molecular detection, *Staphylococcus aureus* virulent *nuc* gene yielded a product of 279bp (Figure 3). Kalorey *et al.* (2007); Turkyilmaz *et al.* (2013) also targeted *nuc*

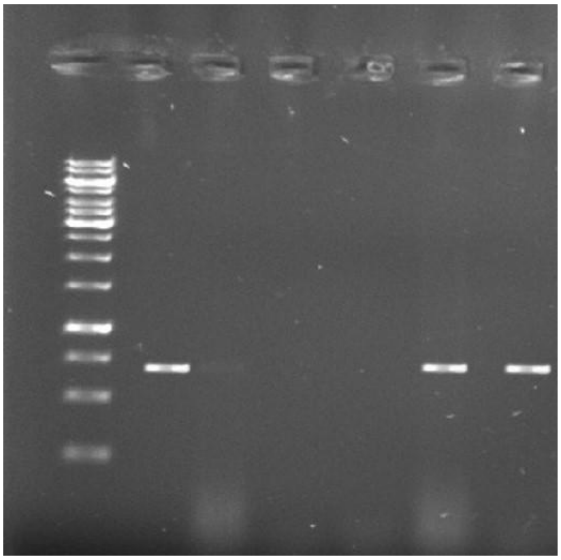

Figure 3: Results for agarose gel showed amplified product at 279 bp indicating *nuc* gene of *Staphylococcus aureus*

gene and found positive results in all pathogenic *Staphylococcus aureus* isolates. Methicillin resistance is caused by the production of penicillin -binding protein, PBP-2a, having decrease binding affinity for β-lactams. The sequence of *mecA* is conserved in all the methicillin resistant strains of *Staphylococcus aureus*. There are two regulatory genes on *mec* DNA, i.e., *mec1* and *mecR1* that control the expression of *mecA* and encode signal transducer protein (Lee, 2003). So that in this study *mecA* gene was targeted. For the molecular detection of MRSA gene (*mecA* gene) yielded product length of 162bp through PCR (Figure 4). Stegger *et al.* (2017) also founded product length of 162bp after targeting methicillin resistant *Staphylococcus aureus mecA* gene.

Oxacillin and vancomycin showed 100% resistant in all the 61 isolates, while enrofloxacin, amoxicillin, ampicillin,

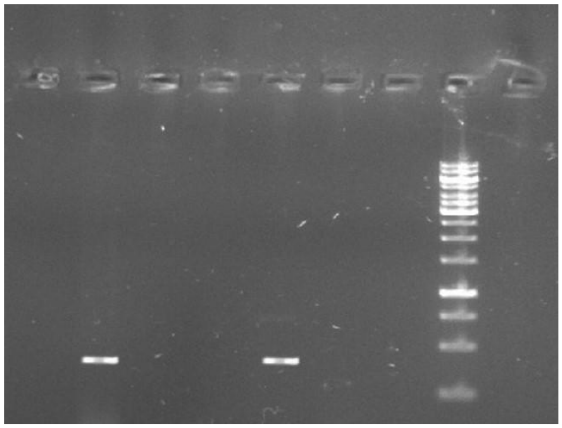

Figure 4: Results for agarose gel showed amplified product at 162 bp indicating *mecA* gene of Methicillin resistance *Staphylococcus aureus*.

oxytetracycline, gentamycin and tylosine showed 1/61 (1.64%), 34/61 (55.74%), 45/61 (73.77%), 8/61 (13.11%), 1/61 (1.64%) and 7/61 (11.74%) resistance, respectively. Susceptibility percentage of enrofloxacin, amoxicillin, ampicillin, oxytetracycline, gentamycin and tylosine were 17/61 (27.87%), 23/61 (37.70%), 9/61 (14.75%), 12/61 (19.67%), 60/61 (98.36%) and 6/61 (9.84%). Enrofloxacin, amoxicillin, ampicillin, oxytetracycline and tylosine showed 43/61 (70.49%), 4/61 (6.56%), 7/61 (11.47%), 41/61 (67.21%) and 48/61 (78.69%), respectively intermediate resistance against MRSA. Maalik *et al.* (2019) found 100% resistance against *Staphylococcus aureus* to Fosfomycin, Kanamycin, Oxacillin, Penicillin and Trimethoprim. Augmentin, Cefoxitin, Clindamycin and Gentamycin showed 92.3% resistance, while Ampicillin, Chloramphenicol, Erythromycin, Ofloxacin, Rifampicin and Vancomycin showed 84.6% resistance (Maalik *et al.*, 2019). In this study, Gentamycin (10µg) was the most susceptible drug. Umaru *et al.* (2013) found amoxicillin, chloramphenico, ciprofloxacin, erythromycin, gentamycin, oxacillin, penicillin, sulphamethoxazole/trimethoprim, tetracycline, and vancomycin resistant to 18 MRSA isolates, while they found 100% susceptibility of amikacin to all the isolates. These results suggest that gentamycin can be used for the treatment of mastitis in Pakistan, but a regular monitory is required.

5. CONCLUSIONS

Among the mastitic cases, both in buffalo and cattle, 58.09% were positive for *Staphylococcus aureus.* These isolates were resistant to most of the drugs tested but were susceptible to gentamycin in 98.36% and tylosine in 78.69% cases. There are many genes of *Staphylococcus aureus* which are responsible for mastitis like coagulase coa, Staphylococcus enterotoxin gene C (sec), staphylococcus enterotoxin gene D (sed),toxic shock syndrome (tsst) gene, we choose the (nuc) which is thermostable nuclease which we come to know is most prevalent genes in Punjab so we decided to target most potent gene in our region so that we can be able to control this problem by making vaccine as it brings huge economic losses so that we can move to vaccine production for the mastitis as there is no vaccine available in field which is successful against mastitis. This disease is big issue in dairy farm and for now we also come to know many antibiotics are resistant against mastitis as our study showed that cure is going to be difficult day by day. After performing antibiotics susceptibility, we come to know about the most suitable combination of antibiotic for its treatment with more effectiveness.

6. REFERENCES

Abo-Shama, U. 2014. Prevalence and antimicrobial susceptibility of *Staphylococcus aureus* isolated from cattle, buffalo, sheep and goats. Assiut. Vet. Med. J. 60:63–72.

Akineden, O., C. Annemuller, A.A. Hassan, C. Lammler, W. Wolter, and M. Zschock. 2001. Toxin Genes and Other Characteristics of *Staphylococcus aureus* Isolates from Milk of Cows with Mastitis. Clin. Vaccine. Immunol. 8:959–964

Ali, M.A., M.D. Ahmad, K. Muhammad, and A.A. Anjum. 2011. Prevalence of Sub Clinical Mastitis in Dairy Buffaloes of Punjab, Pakistan. J.

Anim. Plant. Sci. 21:477-480.

Aqib, A.I., M. Ijaz, A.A. Anjum, M.A.R. Malik, K. Mehmood, S.H. Farooqi, and K. Hussain. 2017. Antibiotic susceptibilities and prevalence of Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from bovine milk in Pakistan. Acta. Trop. 176:168-172.

Atiq. M., Riasat. M., Rajput. N. A., Sahi. S. T., Usman. M., Mazhar. H. M. R., Nawaz. A., Jabbar. A., Rehman. N. U., Yaqoob. F., Shaheen. H. M. U., 2021 Estimation of antibiotics and synthetic chemicals towards bacterial wilt of potato caused by Ralstonia Solanacearum.Agric. Sci. J. 3(2): 51-59.

Becker, K., C. Heilmann, and G. Peters. 2014. Coagulase-negative staphylococci. Clin. Microbiol. Rev. 27:870–926.

Costa, A.M., I. Kay, and S. Palladino. 2005. Rapid detection of mecA and nuc genes in staphylococci by real-time multiplex polymerase chain reaction. Diagn. Microbiol. Infect. Dis. 51:13–17.

Hoekstra, J., A.L. Zomer, V.P. Rutten, L. Benedictus, A. Stegeman, M.P. Spaninks, T.W. Bennedsgaard, A. Biggs, S. De Vliegher, D.H. Mateo, and R. Huber-Schlenstedt. 2020. Genomic analysis of European bovine *Staphylococcus aureus* from clinical versus subclinical mastitis. Sci. Rep. 10:1-11

Joshi, L.R., and S.P. Devkota. 2014. Methicillin Resistant *Staphylococcus aureus* (MRSA) In Cattle: Epidemiology and Zoonotic Implications. Intern. J. Appl. Sci. Biotechnol. 2:29-33.

Kalorey, D.R., Y. Shanmugam, N.V. Kurkure, K.K. Chousalkar, and S.B. Barbuddhe. 2007. PCR-based detection of genes encoding virulence determinants in *Staphylococcus aureus* from bovine subclinical mastitis cases. J. Vet. Sci. 8:151-154.

Kateete, D.P., C.N. Kimani, F.A. Katabazi, A. Okeng, M.S. Okee, A. Nanteza, M.L. Joloba, and F.C. Najjuka. 2010. Identification of Staphylococcus aureus: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. Ann. Clin. Microbiol. Antimicrob. 9:23.

Lee, J.H. 2003. Methicillin (Oxacillin)-Resistant *Staphylococcus aureus* Strains Isolated from Major Food Animals and their Potential Transmission to Humans. Appl. Environ. Microbiol. 69:6489–6494.

Maalik, A., S. Ali, S. Iftikhar, M. Rizwan, H. Ahmad, and I. Khan. 2019. Prevalence and antibiotic resistance of *Staphylococcus aureus* and risk factors for bovine subclinical mastitis in District Kasur, Punjab, Pakistan. Pak. J. Zool. 51:1123–1130.

Mandell, G.L. 1975. Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus*. In vitro and in vivo studies with emphasis on staphylococcal leukocyte interaction. J. Clin. Investig. 55:561–566.

Sperber, W., and S.R. Tatini. 1975. Interpretation of the tube coagulase test for identification of *Staphylococcus aureus*. Appl. Microbiol. 29:502–505.

Stegger, M., P.S. Andersen, A. Kearns, B. Pichon, M.A. Holmes, G. Edwards, F. Laurent, C. Teale, R. Skov, and A.R. Larsen. 2017. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus*. Clin. Microbiol. Infec. 18:395-400.

Turkyilmaz, S., Z. Erdem. 2013. Molecular Typing of Methicillin Resistant *Staphylococcus aureus* Strains Isolated from Cows and Farm Workers. Kafkas. Üniv. Vet. Fak. Derg. 19:963–968.

Umaru, G.A., J. Kabir, V.J. Umoh, M. Bello, and J.K.P. Kwaga. 2013. Methicillin-Resistant *Staphylococcus aureus* (MRSA) in fresh and fermented milk in Zaria and Kaduna, Nigeria. Inter. J. Drug. Res. Technol. 3:67–7