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MOLECULAR CHARACTERIZATION OF *Staphylococcus aureus* ISOLATES RECOVERED FROM NATURAL CASES OF SUBCLINICAL MASTITIS IN CHOLISTANI CATTLE AND THEIR ANTIBACTERIAL SUSCEPTIBILITY

Abdul Qayyum[1], Jawaria Ali Khan[2], Riaz Hussain[1,*], Ahrar Khan[3,*], Muhammad Avais[2], Nisar Ahmad[4] and Mohammad Farooque Hassan[5]

[1]University College of Veterinary and Animal Science, The Islamia University of Bahawalpur-63100, Pakistan;
[2]Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences, Lahore, Pakistan;
[3]Department of Pathology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan;
[4]Department of Parasitology, University of Veterinary and Animal Sciences Lahore, Pakistan.
[5]Shaheed Benazir Bhutto University of Veterinary and Animal Sciences, Sakrand, Pakistan.
[*]Corresponding author's e-mail: ahrar122@uaf.edu.pk (AK); driazhussain@yahoo.com (RH)

Subclinical mastitis primarily with microbial etiology is amenable to different antibiotic therapy and the outcome is doubtful due to the association of a variety of microorganisms, host-specificity and continual evolution of different antibiotic-resistant strains of microorganisms. Therefore, the present study was ascertained to investigate the presence, genotypic characteristics of *Staphylococcus aureus* isolates recovered from subclinically mastitic Cholistani cattle and the effectiveness of different antibiotics against these pathogens by disc diffusion technique. For this purpose milk samples were collected from a total of 1457 lactating Cholistani cattle and screened for mastitis using California Mastitis Test. All the positive samples were processed for culturing. *Staphylococcus aureus* isolates isolated and identified on the basis of colony characteristics, coagulase test, biochemical features and amplification of *spa* (spa-X) and coagulase *(coa)* genes. The results of PCR revealed that amplification of *spa (*spa-X) gene yielded different PCR products (400bp and 350bp) while coagulase (*coa*) produced different products size (390bp, 500bp, and 600bp) indicating genetic variation within and among different herds of the cattle. Moreover, results of this study showed that the *spa (*spa-X) gene present in coagulase positive (179) and coagulase negative (4) *S. aureus* isolates. *S. aureus isolates* were fully sensitive (88%) to amoxicillin, followed by enrofloxacin (78%) and highly resistant to penicillin (65%) and cephradine (100%). It is therefore concluded that *S. aureus* isolates were genetically different in the study areas and amoxicillin is the drug of choice for treating subclinical mastitis.

Keywords: Cholistani cattle, subclinical mastitis, *Staphylococcus aureus*, antibacterial susceptibility, amoxicillin, enrofloxacin, antibiotic-resistance.

INTRODUCTION

Mastitis being the most significant disease of dairy animals largely affects the farm economics by decreasing milk production, and increasing the treatment costs (Mohammadian, 2011; Singh *et al.*, 2015). Mastitis is a multi-etiological disease, however, *Staphylococcus aureus* is the most important and lethal agent (Jaradat *et al.*, 2014) that causes chronic and deep infections in mammary tissue and becomes difficult to treat successfully and it is responsible for dairy scourge in the livestock industry (Hussain *et al*., 2012a; Raza *et al*., 2013). *Staphylococcus aureus* is the most commonly isolated pathogen in subclinical mastitis (Amin *et al*., 2011; Dieser *et al*., 2014; Cengiz *et al*., 2015). Economic significance to dairy business on account of Staphylococcal udder infection results from the subclinical mastitis escorted with a decrease in milk quantity and quality (He *et al*., 2014; Kuçukonder *et al*., 2015). *Staphylococcus aureus* remains a major issue under different tropical and sub-tropical management situations. *Staphylococcus aureus* possesses several proteins which are virulent in nature and transmits simply in lactating animals (Maksymiec and Mikolajczyk, 2012). Coagulase and spa proteins are virulence factors found in *S. aureus* which induces mastitis (Karahan *et al*., 2011) and *spa* is a surface protein of cell wall that impairs the opsonisation and the phagocytosis process by binding with IgG antibody (Gao and Stewart, 2004).

Eradication of *S. aureus* is very difficult despite the conduction of intense measures for control purpose. Consequently, control of the *S. aureus* mastitis has fundamental importance and remains as essential (Waller *et al*., 2009; Hussain *et al*., 2013b; Jaradat *et al*., 2014). Several approaches regarding phenotyping and genotyping procedure are being used to sub-type the *Staphylococcus aureus* isolates recovered both from animals and human (Kalorey *et al*., 2007; Saei *et al*., 2009). The molecular diagnosis could be the most suitable technique for identification of various circulating strains of pathogens which are difficult to identify by

conventional methods. The molecular-based techniques are much effective in pursuing the spreading of bacterial infections and developing the accomplishments of disease control program (Hussain *et al*., 2012b; Mahmmod *et al*., 2013; Qian *et al*., 2014).

Antimicrobials having key role in mastitis control programs and mastitis is major cause of using antibiotics in dairy animals (Awandkar *et al*., 2013). Thus, investigation of antibiotic susceptibility is also an essential to make sure the ideal results of antibiotic's use against the bacterial agents through proper selection on the basis of antibiogram studies (Moroni *et al*., 2006; Awandkar *et al*., 2009). However, indiscriminate use of antimicrobial agents against the udder infection makes it more vulnerable for development of bacterial resistance. It is one of the reasons for treatment failure in mastitis without testing the in vitro sensitivity patterns of therapeutic agents against the causative organisms (Alian *et al*., 2012; Haque *et al*., 2014). Furthermore *S. aureus* attains the antibiotic resistance with remarkable adeptness (Booth *et al*., 2001). Therefore, the present study was conducted to determine the distribution and genotypic characteristics of *S. aureus* isolates and their antimicrobial susceptibility recovered from sub clinically infected milk samples of Cholistani cattle.

MATERIALS AND METHODS

Isolation and confirmation of S. aureus: This study was carried out on a total of 1457 lactating Cholistani cows for sub-clinical mastitis. Lactating Cholistani cows through cluster sampling were included from 71 villages and 27 tobas of the Cholistan in this study. One village/Toba was taken as a single cluster having 10 Cholistani cows in lactation. Lactating cows from public livestock Jugait peer farm were also examined for subclinical mastitis. Milk samples were tested using California Mastitis Test (CMT) following the standard protocol (Schalm *et al*., 1971). CMT Positive samples were subjected to bacterial isolation and identification. For bacterial isolation, a loopful of milk sample was separately cultured on Staph-110 agar medium (Oxoid) and 5% sheep blood agar. After 24 h petri plates were incubated at 37°C and presumptive identifications of *Staphylococci* were carried out based on its colony characteristics, catalase test and coagulase test reactions (National Mastitis Council Inc., 1990). *Staphylococci* were also biotyped through commercially available API 20 Staph kits (BioMerieux, France).

Extraction of bacterial DNA: *Staphylococcus aureus* DNA was extracted from all pure bacterial growths (n=273) obtained on selective staph 110 agar and also on blood agar supplemented with 5% sheep blood. Briefly, 3-4 well defined colonies of pure bacterial growth recovered on the basis of biotyping were mixed in deionized water. The suspension was boiled in water bath for 25-30 min and kept at -20°C for further analysis (Khan *et al*., 2013a).

DNA amplification: Coagulase gene and spa gene typing of *Staphylococcus aureus* was carried out through PCR analysis (Guler *et al*., 2005) with some modifications. Amplification of coagulase gene was carried out by using specific primers: Coag-2 (5-CGA GAC CCA GAT TCA ACA AG-3) as forward and Coag-3 (5-AAA GAA AAC CAC TCA CAT CA-5) as reverse primer. A total of 25 µl volume containing 17 µl of master mix, 5 µl of DNA template, 3 µl of primers mixed thoroughly with help of vortex mixer. For coagulase gene amplification, a total of 35 PCR cycles each consisting of denaturation for 45 seconds at 94°C, annealing for 45 seconds at 50°C and extension for 90 seconds at 72°C were carried out. The initial denaturation was performed at 94°C for 4 minutes. *Spa* gene amplification using primers 5´-GCT AAA AAG CTA AAC GAT GC-3´ and 5´-CCA CCA AAT ACA GTT GTA CC-3´ (Khan *et al*., 2013a) was also subjected to 35 PCR cycles, consisting of denaturation for 45 seconds at 94°C, annealing for 45 seconds at 58°C and extension for 90 seconds at 72°C. The amplified PCR products were run on 0.8% agarose gel electrophoresis for 60 minutes at 90 volts then visualized and photographed under the UV lamp (Sambrook *et al*., 2002).

***Antibiogram studies*:** The confirmed *Staphylococcus aureus* isolates through PCR analysis of coagulase and spa genes were tested for their susceptibility to various antibiotics such as enrofloxacin, ciprofloxacin, norfloxacin, oxytetracycline, penicillin, amoxicillin, ampicillin, gentamicin and cephradine by disc diffusion method (Kirby and Bauer, 1966; Anonymous, 2004).

RESULTS

***Identification of Staphylococcus aureus by polymerase chain reaction*:** The *Staphylococcus aureus* was confirmed in a total 197 (72.2%) from 273 Staphylococcal isolates obtained from 320 CMT positive milk samples initially identified by API 20-STAPH kits. The coagulase gene was confirmed from coagulase test positive (179/194) isolates and also from coagulase test negative (18/79) isolates. In this study, three different PCR products approximately, 390bp, 500bp and 600 bp for coagulase gene (Fig.1) and two different PCR products approximately, 350 bp and 400bp for spa gene (Fig. 2) from *S. aureus* were amplified by using coagulase gene and spa gene primers (Table 1).

***Antibiogram patterns of Staphylococcus aureus isolates*:** The susceptibility patterns of PCR confirmed isolates of *Staphylococcus aureus* were tested against different antimicrobial agents by disc diffusion methods showed that amoxicillin (Fig.3) was highly effective (88%). Highest resistance was recorded against cephradine and penicillin 100% and 65%, respectively (Table 2).

Table 1. PCR based distribution of coagulase and spa genes (n) at different areas of study.

Genes/PCR product (bp)	No (%)	Villages	Tobas	Public Farm
Coagulase (n=197)				
600bp	54 (27.4)	27	24	3
500bp	117 (59.4)	99	13	5
390bp	26 (13.2)	19	6	1
Spa gene (n=183)				
400bp	112 (61.2)	89	18	5
350bp	71 (38.8)	50	19	2

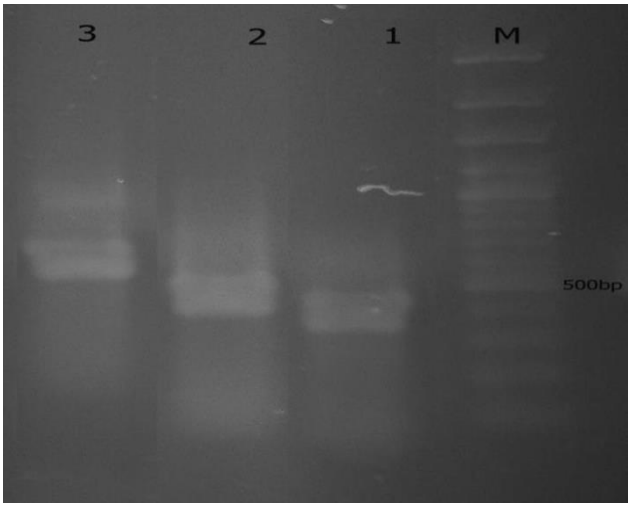

Figure 1. PCR amplification and gel electrophoresis of coagulase gene stand with ethidium bromide. lane 1: 390bp Lane 2: 500bp Lane 3: 600bp ;M: 100bp DNA marker.

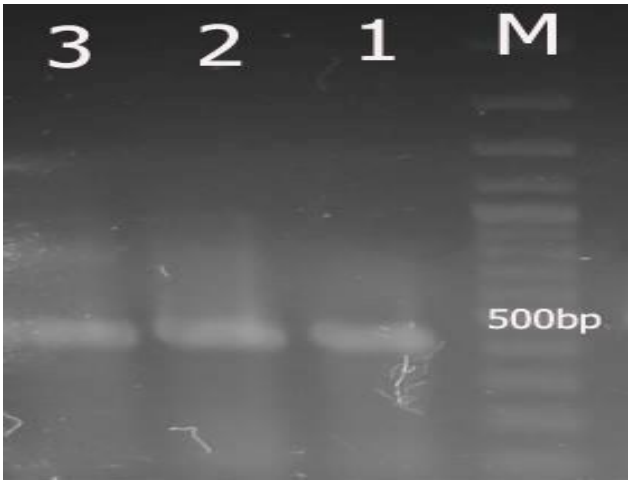

Figure 2. PCR amplification and gel electrophoresis of spa gene (400bp) stand with ethidium bromide; lane 1, 2 and 3 positive samples; M: 100bp DNA marker.

Table 2. Antibiogram patterns of PCR confirmed *Staphylococcus aureus* isolates.

Antimicrobial Agents	µg/disc or Unit/disc	Zone Diameter (mm)	Percent (%)		
			S	I	R
Amoxicillin (AML 10)	10	28-33	88	0	12
Ampicillin (AMP 10)	10	22-25	74	10	16
Norfloxacin (NOR 10)	10	18-25	61	29	10
Enrofloxacin (ENR 5)	5	12-26	78	0	22
Oxytetracycline (OT 30)	30	20-29	56	7	37
Gentamicin (CN 10)	10	14-20	55	45	0
Ciprofloxacin (CIP 5)	5	12-26	70	30	0
Penicillin (P 10)	10	12-30	15	20	65
Cephradine (CR 10)	10	8-11	0	0	100

Sensitive (S); Intermediate (I); Resistant (R)

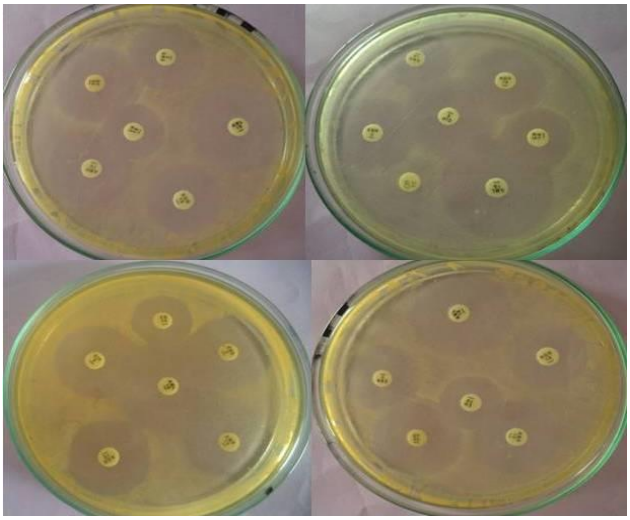

Figure 3. Antibiogram patterns of *Staphylococcus aureus* isolates.

DISCUSSION

The investigation and analysis of intramammary gland pathogens is crucial to control the mastitis. In Pakistan, among the infectious diseases, mastitis is the major threat which causes adverse impacts on milk production and its quality (Hussain *et al*., 2013b; Khan *et al.*, 2013a). Therefore this study was undertaken to investigate the genetic homogeneity and heterogeneity among *S. aureus* isolates recovered from subclinically infected mastitic Cholistani cattle. In present study three different PCR products approximately 390bp, 500bp and 600 bp were obtained from *Staphylococcus aureus* isolates for coagulase genes. Previously in Cholistani cattle no report is available about the molecular investigation of *Staphylococcus aureus* isolates however; limited data is available about the genetic homogeneity and heterogeneity among *S. aureus* in different herds of buffaloes and cattle (Khan *et al*., 2013a). In our study

the size of coagulase gene fragments generated by PCR has also been reported by different previous researchers (Khan *et al*., 2013a) with same coagulase gene primers. However, in contrast to our results a single amplicon of 710 bp size for coagulase gene has been reported (Tyagi *et al*., 2013). Work of Cabral *et al*. (2004) and Coelho *et al*. (2009) also supported our results about different product sizes of coagulase genes who also reported that amplification of coagulase gene showed four different PCR product sizes. Although the exact source for the coagulase gene polymorphism in different *Staphylococcus aureus* bacteria is still unclear. The different PCR product size in this study might be due to the diversity in allelic form of coagulase gene (Goh *et al*., 1992; Aslantas *et al*., 2007). It could be as a result of inclusion or deletion or mutation by which a part of 3' end area of coagulase gene is deleted or added numerous nucleotides and as a result changes the coagulase gene product size and might be possibly antigenic properties of the coagulase enzyme (Saei *et al*., 2009; El-Jakee *et al*., 2010; Khan *et al*., 2013a). On the other hand, it might be as a result of different mutations and antigenic capability of coagulase gene (Himabindu *et al*., 2009).

Spa is a suitable gene to detect the difference amongst the *Staphylococcus aureus* pathogens in a short duration (Lange *et al*., 1999; Reinoso *et al*., 2008; Karahan *et al*., 2011). In present study different *Staphylococcus aureus* isolates produced amplicon size of 350 bp and 400 bp for *Spa* gene. The unpredictability and stability of *spa gene* in present study revealed that analysis of this protein is useful for molecular typing of *Staphylococcus aureus* pathogens as a risk factor in its occurrence (Frenay *et al*., 1996; Zecconi and Hahn, 2000; Suleiman *et al*., 2012). Results of the current study revealed that *Staphylococcus aureus* isolates which showed the amplification against coagulase gene also had spa gene. Differences in amplicon size for spa gene were also been reported (Johler *et al*., 2011; Haran *et al*., 2012). The results of this study indicated that some genotypes of *S. aureus* occasionally occurred in lactating dairy herds. The incidence of such kinds of *S. aureus* genotypes could be less adapted to the udder and might be eliminated from the mammary parenchyma (Joo *et al*., 2001). Previously similar investigations have been reported in Pakistan and different other geographical locations (Shopsin *et al*., 2000; Khan *et al*., 2013a).

Staphylococcus aureus isolates showed amoxicillin to be the most sensitive among all the antibiotics tested which was in accordance with the previous studies (Hussain *et al*., 2012a; Idriss *et al*., 2014). However, Umar *et al*. (2013) reported less susceptibility of *Staphylococcus aureus* isolates to amoxicillin while high resistance against amoxicillin has been reported by Unakal and Kaliwal (2010). The less susceptibility and higher resistance *Staphylococcus aureus* isolates to amoxicillin could be due to the haphazard use of antibiotic. Results revealed that *S. aureus* isolates were least sensitive to cephardine and penicillin-G. Similar findings have been reported (Khan *et al*., 2013b; Mohanty *et al*., 2013). This higher resistance of *Staphylococcus aureus* against penicillin may be attributed to the production of β-lactamase enzyme responsible for inactivation of penicillin (Abera *et al*., 2010). The highest resistance of *S. aureus* isolates against different antibiotics could be due to the reason of prolonged treatments by same antimicrobial agents.

The antibiogram profile of different bacterial isolates indicated that gentamycin, ciprofloxacin, enrofloxacin, tetracycline, Ampicilin and norfloxacin also proved as effective antimicrobials against *S. aureus* isolates in our study. Similar antibiogram patterns have been reported previously (Iqbal *et al*., 2004; Farooq *et al*., 2008; Charaya *et al*., 2014; Patnaik *et al*., 2014). This variability in sensitivity and resistance may be due to frequent and indiscriminate use of the antibiotics in different herds.

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