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Original scientific article

MOLECULAR CHARACTERIZATION OF METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA) ISOLATED FROM BOVINE WOUNDS

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Staphylococcus aureus (*S. aureus*) is a Gram-positive coccus with golden-colored colonies 0.5-1.5 µm in diameter. It is an opportunistic pathogen and colonizes as healthy flora. When the host defense system is breached it provides a source for the introduction of (Methicillin-resistant *S. aureus*) MRSA. The incorporation of the *mec*A gene shift *S. aureus* into MRSA, *mec*A is a primary gene for the confirmation of MRSA, so, it is used as a useful marker to determine Methicillin resistance in *S. aureus*. In this study, we investigated the molecular characterization of *mec*A, Panton-Valentine Leukocidin (*PVL*), and 16S rRNA genes in MRSA to determine diversity, phylogenetic analysis, and multidrug resistance (MDR) of MRSA isolated from chronic bovine wounds. A total of 8 antibiotics were used for MDR profiling and the results obtained are as follows: 100% of MRSA isolates were resistant to Augmentin and Cefipime, 81.8% to Vancomycin and Tetracyclin, 36.4% to Streptomycin and Ciprofloxacin, Azithromycin 54.5 and 0% to Chloramphenicol which warrants that it's the best antibiotic according to this study. Multiplex PCRs were performed for the confirmation of targeted genes and diversity analysis. The diversity of MRSA in the bovine population was 22% (11/50) on the microbiological scale that considered high as compared to reported data. When the PCR of MRSA isolates was performed, there was a unique phenomenon observed i.e., no *mec*A gene was present in 2 isolates 18.18% (2/11) which connotes the importance of molecular methods/PCR for the identification of microbes. The prevalence of the *PVL* gene was 18.18%, comparatively high as compared to previous studies conducted on bovine chronic wounds. When the Sanger sequencing of 16S rRNA of MRSA isolates was performed there was a change of one nucleotide identified (C>T) at position 1031. After performing phylogenetic analysis with *S. aureus* of different countries distinct and separate dendrogram was obtained which differentiates the Pakistani *S. aureus* isolates from other countries.

Keywords: Bovine, MRSA, *Staphylococcus aureus*, Phylogenetic Analysis, Multiplex PCR.

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INTRODUCTION

Livestock is one of the largest agricultural sectors of Pakistan. It plays a vital role in the stability of an economy and gives 56.3% to the agriculture sector and nearly 11% of the agricultural gross domestic product (GDP) of Pakistan. Pakistan stands the fourth position in the world milk production after the USA, China, and India (REHMAN *et al*., 2017). In Pakistan, 30-35 million rural population depends on livestock and herds that manage 40% of the budget. Milk is the major contributor to the country's economy compared to any crop (BURKI et al., 2004).

S. aureus is a Gram-positive bacterium with 0.5-1.5 μm in diameter and approximately 32 species and sub-species (KLOOS *et al.*, 1994). It is non-spore former, non-motile, catalase, and coagulase-positive bacteria. Usually, it is present on the skin, nostrils, hair, and gastrointestinal tract of the animal. It is an opportunistic pathogen that colonizes as healthy flora of the body, whenever an organism's defense system becomes impaired, it starts causing an infection, leading to severity. This healthy flora may cross primary and secondary barriers of the host defense system and act as a source of MRSA through aspiration, shaving, horn abscess, and common lesions (GORDON *et al.*, 2008). It can cause different diseases to humans and animals like pneumonia, chronic skin infections, and mastitis in bovine, ruminants, and pseudo ruminants such as camels. Therefore, it imparts negative characteristics to the dairy industry worldwide (ZUBAIR *et al.*, 2015). It carries genes, i.e., *mec*A/C, that confirms resistance to a potentially mobile genetic element named *Staphylococcal Cassette chromosome mec* (SCC*mec*) (ITO *et al.*, 2001).

There are many different forms of MRSA based on colonial complexes (CC). CC 398 is said to be live-stock-associated MRSA (LA-MRSA) and it is present all over the world now (PETINAKI and SPILIOPOULOU, 2012). When penicillin was not introduced, there was no cure for infections caused by *S. aureus* except carbolic acid which was suggested by Lister and Ogston (MYLES *et al.*, 2012). Penicillin was introduced in 1942 as a wonder drug to combat the problems caused by it. After few years, penicillin-resistant *S. aureus* was isolated. The acquired resistance of *S. aureus* was due to the penicillinase enzyme formed by the microorganisms. As soon as the penicillin became resistant to *S. aureus*, Methicillin was offered to the market formally. The starting point to the resistance against Methicillin was due to the acquisition of the *mec*A gene occurred which we call now MRSA that encodes an enzyme called Penicillin-binding protein 2a (PBP2a) (FIGUEIRDO and FERRERIA, 2014).

MRSA was first isolated in 1960 and soon after its detection it became a major hospital-acquired pathogen (HA-MRSA) which is now prevalent all around the world, causing chronic skin infections to life like septicemia. It possesses a greater risk to host's wounds, thus, increasing the mortality rate (NEOPANE *et al.*, 2018). MRSA *and* its isolates are producing contagious skin infections which are more critical to healing and are costlier treatments as compared to *S. aureus* (JAIN *et al.*, 2016).

The *mec*A gene is the structural determinant encoding PBP2a (Penicillin-binding protein 2a). It is a primary mediator for methicillin resistance in *S. aureus.* (PEREZ-ROTH *et al.*, 2001). There is a high percentage of newly evolving community-acquired (CA- MRSA) which has phage-borne genes that code Pantonn-Valentine leukocidin *(PVL)*. Its biochemical properties can cause apoptosis of the leukocyte cells. It can also cause soft tissues and skin infections with necrotizing pneumonia warranting more severe septicity control and therapy control than applied for *PVL* negative *S. aureus* (SOHAIL *et al.*, 2018). It accounts for one of the important virulence

factors, can cause pneumonia, necrosis, white blood cell destruction (BHATTA *et al.*, 2016). It is also responsible for endocarditis, septic arthritis, and empyema. For the treatment of such severe kinds of infections, Vancomycin was employed to market after Methicillin but again *s. aureus* became resistant and the first Vancomycin-resistant *S. aureus* was isolated in 2002 (SHITO *et al.*, 2006). There are certain other elements involved in this process: toxic shock syndrome toxins, hemolysins, and leukocidins, etc. *S. aureus* reflects a major threat to livestock species because of its high prevalence approximately 48% reported. It makes resilient films on wounds hence they become chronic and due to this reason animal become feeble and ultimately reaches to economic loss in yielding milk and meat (TIWARI *et al.*, 2016).

MRSA has high genetic variability and a different number of strains that evolve resistance to antibiotic susceptibility (MADZGALLA *et al.*, 2016). Some strains of it, have gained resistance against most of the antibiotics due to their massive dispersal and incorrect dosage regime, it is an organism that can cause infection in both humans and animals at the same time. In the last few decades, it has been reported in many species as carriers like cats, cattle, and dogs (WAQAR *et al.*, 2019). They are biofilm producers, it protects the microorganisms from host defenses, resists the antibiotics thus impedes wound healing process which may cause chronicity of wounds (NEOPANE *et al*., 2018). Recently, the expansion of antimicrobial agents at a narrow or lean scale has worsened the situation and increased the need for research to discover alternative treatments as a substitute or to replace antibiotics (JENKINS *et al.*, 2013).

MATERIALS AND METHODS

Sample Collection and Processing

A total of 50 samples were collected using sterile cotton swabs from 4 different sites (including veterinary clinics and animal's markets) of Punjab province in Pakistan. Consent from animal owners and a brief history about the time of wound was taken. Alcohol wipes were used to remove bacteria from the skin's healthy flora before taking the sample. The skin lesion content was absorbed by the trans- swabs, inserted into the tube with the transport medium, sealed again, and shifted to the Microbiology department of UVAS, Lahore for processing.

Bacterial Isolation and Identification

For the growth of *S. aureus*, Mannitol Salt Agar (MSA) was used. A sterile loop containing the growth of wound samples has been streaked on MSA plates and incubated at 37°C for 24 hours. *S. aureus* strains were confirmed by using conventional microbiological or biochemical tests, like microscopy, catalase, and coagulase assays and mannitol fermentation, etc. For the identification of *Staphylococcus aureus*, all the necessary biochemical tests were used. Bacterial morphological and microscopic studies were performed. Colony morphology was observed on Mannitol Salt Agar (MSA) and shape was observed by Gram's staining method. 8 biochemical tests such as Catalase, Oxidase, Coagulase, Vogues Proskauer and Methyl red, etc. were performed for the identification of *S. aureus.*

Catalase Test

While carrying out this test, 3% hydrogen peroxide was prepared. A loop full of bacteria was taken from an 18-24 hours MSA plate and inoculated into a drop of 3% H2O2 on a

glass slide and observed for bubble formation, which indicates positive results. This test was made done to check the presence of catalase enzyme that is produced by a series of microbes that tend to live in an oxygenated environment and neutralizes the toxic form of oxygen and *S. aureus* is catalase positive.

Oxidase Test

The oxidase test was performed to identify whether a microbe is a cytochrome c oxidase producer or not. The redox reagent tetramethyl-p-phenylenediamine (TMPD) was prepared fresh for every time use. To carry out this test strip of Whatman filter paper was dipped for a few seconds in a redox reagent. The bacterial colony was taken with the help of a sterile loop and rubbed onto the soaked paper, and results were noted after a few seconds.

Coagulase Test

For this test, rabbit plasma dilutions were prepared in normal saline, and 0.5mL of diluted plasma was poured in autoclave Eppendorf tubes. Isolated colonies of *S. aureus* then picked with a sterile platinum loop and mixed gently into the plasma solution in an Eppendorf and incubated for 4 hours at 37˚ and results were recorded afterwards. This test is critical and plays a vital role in the determination and identification of this bacterium. It was performed to determine the presence of a *staphylocoagulase* enzyme that reacts with a thrombin-like molecule called CRF (Coagulase releasing factor) and combine it to convert fibrinogen into fibrin.

Indole Test

Indole is used to determine the conversion of tryptophan into indole by several intracellular enzymes called "tryptophanase." For this test, 1.5% peptone water was prepared, poured in tubes, and autoclaved. When autoclaving is done, let the tubes cool at room temperature. Then bacterial cultures were inoculated with the help of a sterile platinum loop separately and incubated at 37˚C for 24 hours. After the incubation is done, a few drops of Kovac's reagent (Para-dimethylamino aldehyde alcohol, concentrated hydrochloric acid, and alcohol) were added, and results were noted after a few seconds. *S. aureus* is an indole negative bacterium.

Methyl Red Test

This test is used to determine the ability of microbes to either produce stable acid end-products upon the supply of glucose or not. To carry out this test, MR-VP broth was prepared according to the given instructions on the bottle, poured 10 mL in each tube, and autoclave. With the help of a sterile loop, bacterial culture was inoculated to the tube containing broth and incubated overnight at 37˚C. After the incubation is over, 3-4 drops of MR reagent were added to the tubes, and results were noted after one minute for color change from brown to cherry red, which indicates positive results.

Voges Proskauer Test

Voges Proskauer test is used to analyze the ability of bacteria, whether it is an acetyl methyl carbinol producer or not. Acetyl methyl carbinol is a neutral end product for this purpose

VP broth was prepared first of all according to the instructions on the media bottle, poured 10 mL in each tube, and autoclaved. After the sterilization of broth, a loop full of bacteria was inoculated into the tube carefully near the flame. A tube was capped again, and this process was repeated every time inoculation. After giving inoculum to all the tubes they were incubated at 37˚C for 48 hours, and results were recorded.

Citrate Utilization Test

A citrate utilization test was carried out to check out the bacterial ability if it utilizes citrate as a source of energy or not. For this test, Simmons' citrate agar was prepared according to the manufacturer's instructions, poured in tubes, and autoclaved. To make slant tubes were placed in a tilted position when autoclaving is completed. When the media is fully solidified, then the bacterial cultures were streaked on agar and incubated for 18-24 hours at 37˚C, and the results were cataloged.

Identification of MRSA by Sensitivity Test

First of all, Petri plates were covered in paper and taken to a hot air oven for 1 hour at 170˚C for sterilization. Nutrient agar was made according to the manufacturer's protocol autoclaved and poured into Petri dishes when cooled at room temperature for a while. Plates were incubated at 37˚C overnight to check if there is any contamination. After complete satisfaction, plates were used further for the sensitivity test. 0.5% McFarland was prepared for the preparation of inoculum of 10 ml and turbidity was matched with 0.5 McFarland and adjusted. For bacterial lawn formation on nutrient agar plates, sterile commercially prepared cotton swabs were used for a biological grade. Cotton swabs were immersed in already turbidity adjusted test tubes containing bacterial cultures, pressed with walls of the tube to remove excess material, streaked on nutrient agar plates in a four-way direction, and at the end on the walls of plates in a circular way to ensure equal distribution of inoculum and complete bacterial lawn formation on the entire pate. The cultures were added with the help of a sterile loop near flame very carefully to avoid contamination, Cefoxitin disk (30µg) was used for this test, for the application of this disk sterile forceps were used. Disks were applied in Biosafety Cabinet Level 2 (BSL 2) in the center of inoculated nutrient agar plates and incubated overnight at 37˚C. Before doing any work, the cabinet was cleansed with 70% ethanol then and adequately turned UV lights on for half an hour to ensure complete microbial decay. After incubation on night, the zones of inhibition were measured using the scale in mm. *S. aureus* was declared Methicillin-resistant or Methicillin susceptible according to the zone of inhibition measured. For the declaration of MSSA or MRSA, CLSI standards were kept in view.

Multi-Drug Resistance (MDR) Profile Of MRSA

An antimicrobial sensitivity test was performed to check the multi-drug resistance of all MRSA isolates. For this purpose, nutrient agar plates were prepared according to the manufacturer's protocol. Standard saline tubes were prepared, inoculated with freshly prepared cultures of pure MRSA isolates and adjusted their turbidity by comparing the tubes with 0.5 McFarland standard. By using the sterile cotton swabs carefully immersed them into the already

prepared normal saline tubes and swabbed gently to all four-direction leaving no space after that applied eight antibiotic discs with the help of sterile forceps viz, gentamycin, cefoxitin, augmentin, levofloxacin, ciprofloxacin, cefepime, vancomycin, and ampicillin. After the application of antibiotics was placed the plates shifted in an incubator at 37˚C overnight and results (Growth and zone of inhibition) were cataloged according to CLSI guidelines.

Molecular characterization

DNA Extraction

DNA extraction was done by using Trizol reagent. First, added 250µL of Trizol reagent in a microcentrifuge tube, added loop full of bacterial culture and twisted to and fro to mix the culture, then vortexed the Eppendorf tube for 15 sec. The incubation of samples was done at 70˚C for 30 sec and then short spin. Centrifuged the samples at 12000 rpm for 12 min at 4˚C, transferred the supernatant to a new microcentrifuge tube, added 200µL chilled Isopropanol, vortexed for 30 sec and short spin. The mixture was loaded in the extraction column and centrifuged at 8000 rpm for 1 min. washed the column with 70% ethanol and a quick turn at 8000 rpm for 30 seconds, spin again at 13300 rpm for 2 min and 30 seconds at 4℃. Changed the washing tubes with new Eppendorf tubes and loaded 50µL elution buffer (Low TE buffer). Waited for 1 min centrifuged again at 8000 rpm for 1 min and stored the DNA at -20˚C.

DNA quality and quantity both were measured using Nanodrop and Gel electrophoresis methods. The quality of isolated DNA was checked by taking absorbance at 280 nm along 260 nm. Subsequently, the 260/280 ratio was determined. DNA quality in terms of intact genomic DNA was also described on 1% agarose gel electrophoresis.

Multiplex PCR for the Confirmation of mecA, PVL, and 16S rRNA Gene

The primers used in the current research were earlier reported by (Velasco *et al*. 2015). The primers, as mentioned above, detected the presence of *mec*A, Panton-Valentine Leukocidin *(PVL)*, and 16s rRNA gene. The product size 310 bp represented *mec*A, 433 bp *PVL*, and 756 bp 16S rRNA genes. PCR amplification was carried out using primers, as described in Table 3.2. PCR amplified products of all three genes were determined by using three forward primers and three reverse primers. The primers produced 310 bp, 433 bp, and 756 bp fragments indicating the presence of *mec*A, *PVL*, and 16S rRNA genes, respectively. The master mixture was prepared by mixing the PCR reagents. After the master mixture was made, 25 µl of the master mixture was pipetted into the reaction tube for each PCR. After that, all reaction tubes were transferred into the PCR system to perform PCR amplification.

Sequencing and Phylogenetic Analysis

Once the genes have been confirmed 16S rRNA was used for the sequence analysis and phylogenetic studies

RESULTS

After performing all the required biochemical and normal routine tests 21 *S. aureus* were isolated from 50 samples. Out of these 21, only 11 MRSA were identified by using the sensitivity test. When the Cefoxitin sensitivity test (fig 1) was performed, and results were calculated according to CLSI standards. The minimum value of the Zone of inhibition (0mm)

indicates the resistance while the maximum value of the zone of inhibition (21mm) in the interpreted table indicates the susceptibility (Table 1, Fig 1).

Table 1. Cefoxitin test for the confirmation of MRSA

Sr. no.	Sample code	Zone of inhibition (mm)
1	UF1	9
2	UF4	28
3	UF7	Nil
4	UF10	31
5	UF11	Nil
6	UF13	27
7	UF18	24
8	UF21	30
9	UF23	19
10	UF26	33
11	UF27	13
12	UF29	25
13	UF34	11
14	UF35	27
15	UF37	30
16	UF38	24
17	UF40	11
18	UF42	19
19	UF45	25
20	UF47	16
21	UF50	21
Total MRSA whose zone below 21	11	
% of total isolates	22%	

Figure 1. *S. aureus* resistant to cefoxitin disc

For the determination of multi-drug resistance activity of MRSA, there were eight antibiotics Augmentin (AUG), Cefipimie (FEM), Tetracycline (TE), Streptomycin (S), Vancomycin (VA), Ciprofloxacin (CIP), Azithromycin (AZM), and Chloramphenicol (C) used. Their zone of inhibition was measured and analyzed according to CLSI parameters. Results were

incorporated below (Table 2 and Fig. 2). After multidrug resistance profiling percentage resistance of each bacterium was calculated (Table 3 and Fig. 3).

Table 2. Multidrug resistance profile results

Sr. no.	Bovine Isolates	Antibiotics							
		AUG	FEM	VA	TE	S	CIP	AZM	C
1	**UF 1**	R	R	R	R	I	I	R	S
2	**UF 7**	R	R	I	S	I	R	R	S
3	**UF 11**	R	R	R	R	I	S	R	S
4	**UF 18**	R	R	R	R	I	S	R	S
5	**UF 23**	R	R	R	R	R	I	I	S
6	**UF 27**	R	R	R	R	R	I	S	S
7	**UF 34**	R	R	R	R	I	R	R	S
8	**UF 40**	R	R	R	R	R	I	I	S
9	**UF 42**	R	R	R	R	S	R	I	S
10	**UF 47**	R	R	R	I	R	R	R	S
11	**UF 50**	R	R	I	R	S	I	I	S

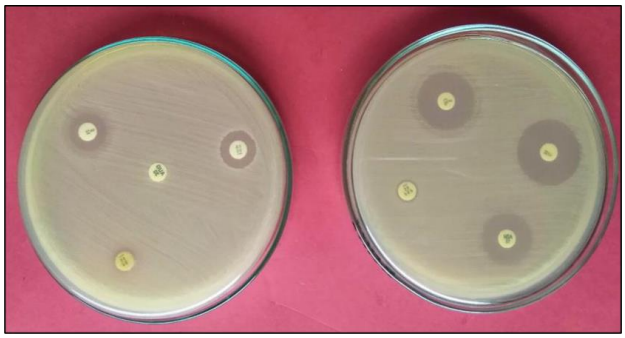

Figure 2. Multi drug resistance activity of MRSA

Table 3. Total MRSA isolates and their resistance percentage value

Antibiotics	Concentration used (µg)	No. of resistant isolates (%age)	Percentage of resistance (%age)
Augmentin	30	11	100
Cefipime	30	11	100
Vancomycin	30	9	81.8
Tetracyclin	30	9	81.8
Streptomycin	10	4	36.4
Ciprofloxacin	5	4	36.4
Chloramphenicol	30	0	0
Azithromycin	15	6	54.5

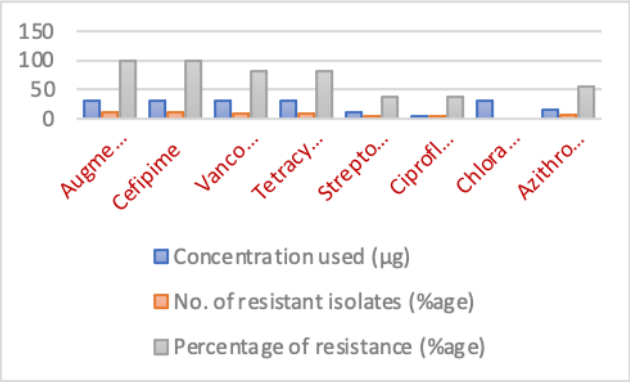

Figure 3. Represents the percentage of resistance against each antibiotic used

Molecular Characterization of MRSA

For the molecular analysis, first, the *mec*A gene was identified in all the MRSA isolates which were not present in two samples, and then *PVL* and 16S rRNA genes were being confirmed by using the gradient PCR technique. After condition optimization multiplex PCR (mPCR) of all isolates was performed.

DNA Extraction of MRSA

Deoxyribonucleic acid of MRSA was extracted from 24h fresh culture grown on TSA agar by using organic method (Trizol reagent) and then it was being run on 1% agarose gel in electrophoresis tank for 30 min at 120 V and observed under gel documentation system to check the quality and quantification was done by using Nanodrop TM 2000.

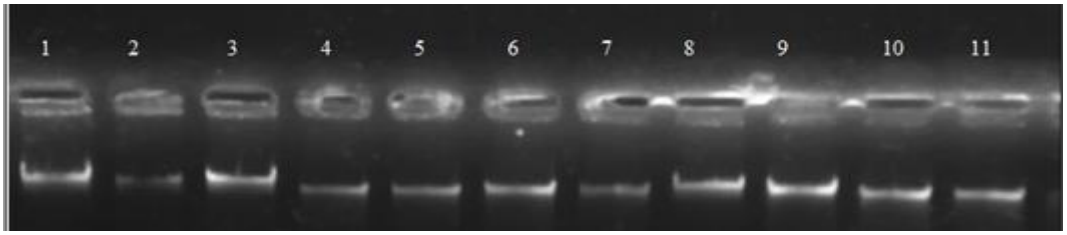

Figure 4. Extracted DNA of all samples

Detection of mecA, PVL, and 16S rRNA genes

Isolated samples of DNA were amplified by using three different regions of MRSA viz., 16S rRNA, *PVL*, and *mec*A genes. For each specific region forward and reverse previously described primers were used (Table 4) for the amplification of gene amplification was done in BIO-RAD T100 Thermal cycler.

Table 4. Set of primers used

Serial no.	Primer	Product size bp	Sequence (5'-3')
1	Staph 16S F	756	5-AACTCTGTTATTAGGGAAGAACA-3
2	Staph 16S R		5-CCACCTTCCTCCGGTTTGTCACC-3
3	Luk PV F	433	5ATCATTAGGTAAAATGTCTGGACATGATCCA-3
4	Luk PV R		5-GCATCAAGTGTATTGGATAGCAAAAGC-3
5	*mec*A F	310	5-AGC ACC CTG GTG GCC AA-3
6	*mec*A R		5-CCACCTTCCTCCGGTTTGTCACC-3

Results of Multiplex PCR

To identify and detect the total no. of genes of interest present in each sample multiplex PCR of all samples was performed and it was identified that the *mec*A gene was present in 81.18% samples, 16S rRNA 100%, and *PVL* gene was available in only 18.18% isolates (fig 5).

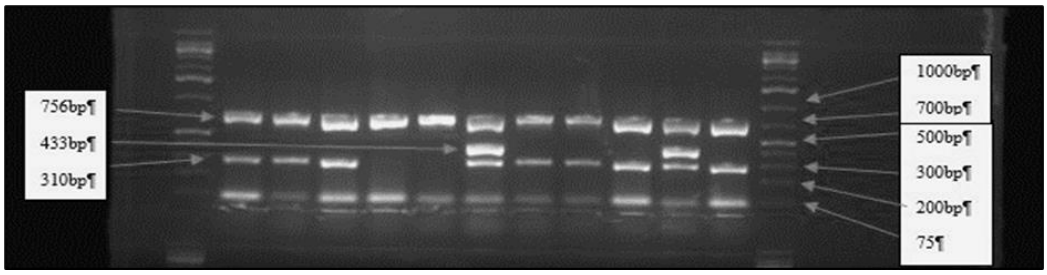

Figure 5. Agarose gel electrophoresis with PCR amplicons of *mec*A, *PVL* and 16S rRNA genes where M is marker of 1 Kb, 16S rRNA gene 756 present in all wells, *PVL* gene 433bp present in well 6 and 10 whereas, *mec*A gene 310bp is present in all wells except 4 and 5

Sequencing results of 16S rRNA gene of MRSA

Sequencing results of MRSA samples were performed and analyzed by using Bio-edit sequence alignment software to find out SNPs. Following is the reference sequence no. of 16S rRNA NR_118997.2. Used against these samples. One mutation was identified at position 1031 where C>T.

Phylogenetic analysis

The phylogenetic tree was made to determine the diversity among different reported sequences from the world and the results were interpreted as follows.

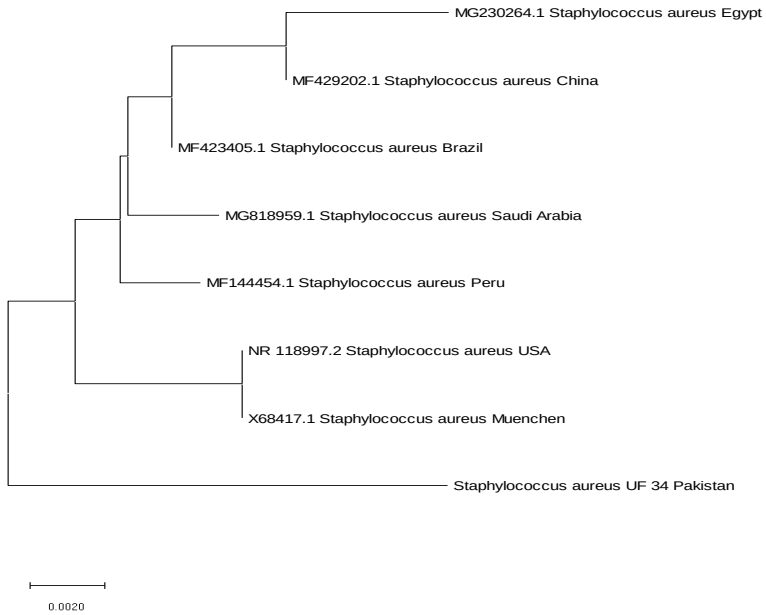

Figure 6. Evolutionary relationships of taxa the evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [2] and are in the units of the number of base substitutions per site. This analysis involved 8 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1558 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [3]

DISCUSSION

Staphylococcus aureus (*S. aureus*) is a natural inhabitant of the normal flora of the nasal cavity and skin. *S. aureus* is an opportunistic bacterium whenever it gets a chance to invade through injuries or mechanical abscissions it leads to the chronicity of wounds by developing an extracellular polysaccharide (EPS) matrix. The MRSA has constantly been a leading cause of wound infections and its slime-producing activity makes it resistant to a wide range of

antimicrobial agents adding to the chronicity of wounds. The *mec*A gene is a structural determinant encoding PBP2a (penicillin-binding protein (2a) and a key mediator for methicillin resistance in *S. aureus*. Thus, *the mec*A gene is used as a helpful predictor to classify MRSA (PEREZ-ROTH *et al.*, 2001). The virulence factor belonging to the family of synergohymenotropic toxins was described by PANTON and VALENTINE (1922) as the *PVL* gene (PANTON *et al.*,1932). The prevalence of the *PVL* gene varies, it was only 9% in Algeria reported in one study (BENHAMED *et al*., 2013). On other hand, it supposed to be higher in MRSA isolates from humans as compared to bovine animals, a study carried out in a hospital reported 56.8% prevalence of *PVL* gene in MRSA isolated from human pus samples, whereas, it was 6.67% in bovine as compared to a human that was 63.63% (BHATTA *et al.*, 2016; PAJIC *et al*., 2014). According to one study that was conducted in Madhya Pradesh, India, the presence of *PVL* gene was 10.5% among MRSA isolated from mastitic milk samples (bovine) (SHRI VASTAVA *et al*., 2018) in another comparative study conducted by PRASHANTH *et al*. (2011) the prevalence of MRSA was 77% among samples isolated from human and 29% among samples isolated from Bovine source. The important thing was that there were no *PVL* positive species found in a single sample (0/34) (PRASHANTH *et al*., 2011). The highest known value of MRSA in a human was identified in India, which was almost 85.1% (KAUR *et al.*, 2012).

In the current study, 2 MRSA isolates lack the *mec*A gene out of 11 (18.18%) and this scenario was also described by BIGNARDI (1996), who identified the inherent resistance of *mec*A genes in the strains. This non-*mec*A *mec*hanism may be due to changes to penicillin that cause further β-lactamase or methicillinase production (BIGNARDI *et al.*, 1996). This phenomenon is explained by Elhassan *et al* in 2015 that there was no *mec*A gene in 12/123 isolates 9.8% (ELHASSAN *et al.*, 2015). There is no evidence yet for a less clinically significant non-*mec*A resistance as MRSA is still present (SWEDSON *et al.*, 1996). In this study, there are only two isolates that were *PVL* positive (18.18%) which is a high prevalence as compared to reported data of many countries. Its prevalence is unpredictable and variable in regions of the world, i.e. very low to very high for example according to one study conducted in Serbia the *PVL* gene was present only in 5 out of 75 (6.6%) isolates extracted from bovine wounds and in human 63% approximately (PAJIC *et al.*, 2014). Many epidemiological studies focused on the high rate of MSRA associated with medical infections worldwide. There are endemic and epidemic strains in each geographic region (EHSANOLLAH GHAZNAVI-RAD *et al.*, 2018).

Multidrug resistance is the key feature of *S. aureus* it was investigated in this study and the following results were obtained: eight antibiotics Augmentin (AUG), Cefipimie (FEM), Tetracycline (TE), Streptomycin (S), Vancomycin (VA), Ciprofloxacin (CIP), Azithromycin (AZM) and Chloramphenicol (C) used. Their zone of inhibition was measured and analyzed according to CLSI parameters. All MRSA were resistant to Augmentin and Cefipime (100%), 81.8% to Vancomycin and Tetracyclin, 36.4% to Streptomycin and Ciprofloxacin, Azithromycin 54.5, and 0% to Chloramphenicol which means it's the best antibiotic according to this study. This MDR experimentation on bovine animals is also performed by TIWARI *et al*. (2016) in Uttar Pardesh India and the results of the study revealed maximum sensitivity to gatifloxacin (94.20%) and 100% resistance against kanamycin, colistin, clindamycin, penicillin G, cotrimoxazole, and cefotaxime. Some studies showed *S. aureus* 100% resistance to methicillin but in this study, it is 52% (TIWARI *et al.*, 2016).

For the identification of MRSA, it was objected to molecular level confirmation based on the presence or absence of the *mec*A, *PVL*, and 16S rRNA gene by PCR testing. First, the *mec*A gene was identified in all the MRSA isolates, which was not present in two samples, and then *PVL* and 16S rRNA genes were being confirmed by using the gradient PCR technique. After condition optimization multiplex PCR (m-PCR) of all isolates was performed and has shown ideal results i.e. 16S rRNA gene present in all 11 samples, *PVL* in 2 and *Mec*A in 9 isolates, all samples were multidrug resistance except 2. Sanger sequencing of the 4 isolates was performed it was identified that there was a change of one base pair in one isolate when compared to the reported data (ATCC 12600) available on NCBI by using the Bioedit, Clustal W, MEGA X software, and BLASTn.

Resistant genes forming ability of *S. aureus* leads to antibiotic resistance and hence, delays in wound healing resulting in chronic wounds. Due to the emergence of antibiotic sensitivity and the lean development of new potent antibiotics, MRSA is becoming a severe threat. Therefore, some alternative treatments/methods are required actual to control MRSA infections. The chronic infections caused by MRSA in bovine have not been previously well-characterized molecularly and documented in Pakistan. Therefore, more investigations are still needed to gather the data of the entire country.

CONCLUSION

Multidrug resistance, sequencing, and phylogenetic analysis were performed of MRSA isolated particularly from bovine chronic wounds. The prevalence of MRSA in bovine chronic wounds was found to be 22%, i.e., 11/50 on the microbiological scale. But, when further investigated and expanded our study to the molecular level, there was no *mec*A gene present in 2 isolates which connotes the importance of molecular methods/PCR for the identification of microbes. Moreover, the more important thing observed was the prevalence of *PVL* gene 18.18%, which is high as compared to most of the other studies conducted on bovine wounds in the world and when the sequencing of MRSA samples was performed one mutation at position 1031 (C>T) of 16S rRNA was identified.

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MOLEKULARNA KARAKTERIZACIJA *Staphilococcus aureus* (MRSA) REZISTENTNOG NA METICILIN IZOLOVANE IZ GOVEĐIH RANA

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Izvod

Ovo istraživanje je sprovedeno u periodu 2011-2013. godine na populaciji duda provincije *Staphilococcus aureus* (*S. aureus*) je gram-pozitivna koka sa kolonijama zlatne boje prečnika 0,5-1,5 μm. To je oportunistički patogen i kolonizuje se kao zdrava flora. Kada je sistem odbrane domaćina narušen, on predstavlja izvor za uvođenje (*S. aureus* otporne na meticilin) MRSA. Inkorporacija mecA gena pomera *S. aureus* u MRSA. mecA je primarni gen za potvrdu MRSA, pa se koristi kao koristan marker za određivanje rezistencije na meticilin kod *S. aureus*. U ovoj studiji smo istražili molekularnu karakterizaciju gena mecA, Panton-Valentine leukocidina (PVL) i 16S rRNA gena u MRSA da bismo odredili raznolikost, filogenetičku analizu i rezistenciju na više lekova (MDR) MRSA izolovane iz hroničnih rana goveda. Za profilisanje MDR korišćeno je ukupno 8 antibiotika i dobijeni rezultati su sledeći: 100% MRSA izolata je bilo rezistentno na Augmentin i Cefipim, 81,8% na vankomicin i tetraciklin, 36,4% na streptomicin i ciprofloksacin, 54,5% na azitromicin i 0% na hloramfenikol što garantuje da je to najbolji antibiotik prema ovoj studiji. Multipleks PCR je urađen za potvrdu ciljanih gena i analizu diverziteta. Raznovrsnost MRSA u populaciji goveda bila je 22% (11/50) na mikrobiološkoj skali koja se smatrala visokom u poređenju sa prijavljenim podacima. Kada je urađen PCR izolata MRSA, primećen je jedinstveni fenomen, tj. u 2 izolata (18,18% - 2/11) nije bio prisutan mecA gen što ukazuje na značaj molekularnih metoda/PCR za identifikaciju mikroba. Prevalencija *PVL* gena bila je 18,18%, relativno visoka u poređenju sa prethodnim studijama sprovedenim na hroničnim ranama goveda. Kada je izvršeno Sanger sekvenciranje 16S rRNK izolata MRSA, utvrđena je promena jednog nukleotida identifikovanog (C>T) na poziciji 1031. Nakon filogenetske analize *S. aureus* iz različitih zemalja dobijen je različit i poseban dendrogram koji razlikuje pakistanski izolat *S. aureus* od drugih zemalja.

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