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Molecular Study of Some Virulence Genes in Biotype Diversity of Methicillin Resistance *Staphylococcus aureus* isolated from Handling Carrier and Bovine Mastitis

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Abstract: ***Methicillin-resistant Staphylococcus aureus (MRSA) more serious problem in dairy animals suffering from mastitis. In this study collated 130 sample of mastitis milk and 130 from nasal worker. The S. aureus characterized phenotypically by biochemical and API Stphand genotypically by used sua gene specific species. (47.69%) percentage of isolation in mastitis milk, (82.25%) biotype C and (4.83%) biotype B that origin from bovine and (12.90%) biotype A origin from human, while isolation S. aureus(44.61%) from nasal carrier, the biotype A recoded 91.37% and 8.63% biotype C origin from bovine. The isolates were tested using agar disc diffusion method for oxacillin and cefaxiton and confirmatory mec A gene by PCR(28.12%) was appeared MRSA from bovine and (35.48%) from human isolated, all of MRSA have all of virulence genes ctfA gene, Spa gene and Nuc gene for their antimicrobial susceptibility to 10 different antimicrobial drugs. Most of MRSA isolates were found to be multi-drug resistant.***

Keywords: biotypes, *S. aureus* MRSA

1. Introduction

S.aureus is considered as a significant pathogen with related virulence factors such as slime factor (biofilms), PVL (Panton Valentine Leucocidine) and some enzymes (proteases, lipases, and elastase), which enable it to destroy host tissues and metastase to other sites.[1]MRSA infection from mastitis cases is partly related with failure in dosage therapy and choice of antimicrobial substance.[2, 3]

Methicillin-resistant *S. aureus* (MRSA) includes *S.aureus* have acquired gene, called *mec*A, this gene encoding to resistance of methicillin and possibly to some beta-lactam antibiotics. MRSA was first reported as a nosocomial pathogen in 1961, soon after methicillin was introduced into human medicine to treat penicillin-resistant staphylococci.[4]

MRSA clones have particularly been detected in animal populations.[3,5] Although MRSA is mostly associated with the acquiring *mec*A gene, the role of inappropriate antibiotics use should also not be under estimated in formation of bacterial resistance and multidrug resistant strains.[6] MRSA infection from mastitis cases is partly related with failure in dosage therapy and choice of antimicrobial substance.[2, 3]

The emergence of (MRSA) poses a serious public health threat. Strains of resistant to b-lactam antibiotics are known as (MRSA).[7]Resistance of methicillin is caused by the gain of the *mecA* gene. PBP2A gene encodes for alternative Penicillin-binding protein, which has a low affinity for beta-lactam antibiotics.[8]SCCmec Staphylococcal Cassette Chromosome mec , *mecA* gene one of a large movable genetic. MRSA are acquired multidrug resistant,MRSA have been give an account to resist most antibiotics like macrolides, fluoroquinolones, chloramphenicol, tetracycline and aminoglycosides.[9]

Commercial identification panels such as Vitec, Sensititre, BACTEC, API-*Staph* identification systems have been used to identify *Staphylococcus* to sub-species inboth human and animal isolates for many years.[10]*Mec*A detection by PCR is accepted as the gold standard method for identifyingMRSA.[4]

In the control of mastitis, the improper use of antimicrobial agents on dairy farm animals is a major concern as it leads to the emergence of resistant zoonotic bacterial pathogens.[11]

In veterinary medicine the extension with treatment the antibiotics are used for some different purpose such as enhance efficiency of feeding and enhance growth of animals. This different purpose enhance multiple drugs resistant to zoonotic bacterial pathogens dissemination.[12, 13]

Community-acquired infections in every region of the world is associated with *S. aureus* recognized as causing health care. *S. aureus* that able to produce Enterotoxins in milk possess a potencyriskiness to healthy of consumers.[14]

The aims of this study was isolation, characterization, biotyping of *S. aureus* and study some virulence molecular detection of MRSAfrom cases of mastitis milk mastitis and worker or cattleman handling with infected cattle and determine the reservoir for MRSA infection cattle and humans and vice –versa.

2. Materials and Methods

Samples collection, this study was performed between October 2013 and July 2015 from Waste Province. Milk samples (130) CMT was scored between I-IV isolation of staphylococcus was attempted depend on clinical sign and decrease milk production were tested by California mastitis test (CMT) were graded as negative, trace, weak, distinct, or strong positive, (clinical and subclinical mastitis).[15]Worker

sample collect from human handling with bovine, 130 samples were takenby nasalswab under sterile conditions for further bacteriological analysis.

The specimens were inoculated onto Mannitol salt agar MSA Himedia - India; and primary cultures incubated at 37°C for 24 h. All colonies were purified by sub culturing onto MSA medium.[16]

Biochemical testes

The identification of the bacteria was performed by a tube coagulase test, mannitol fermentation on Mannitol salt agar and coagulase test tube and detection of clumping with plasma on slide, the production of hemolysis of isolates was determined by cultivation of the bacteria on blood agar plates and vogues proskuor VP, urease oxidase Latex agglutination (MASTSTAPH); considered as *S. aureus* with finally detection by API Staph.[17, 18, 19]

DNA isolation

KAPA Express Extract KK7100 (50 rxns) Transfer DNA containing supernatant to a fresh tube. And may be diluted in TE buffer for long term storage at -20. (KAPA BIOSYSTEMS).

Genotypic characterization

Kit KAPA Taq Ready Mix DNA polymerase contain Taq DNA polymers (0.0 5U /1.25 U per 25 ul) Reaction buffer with Mg $^{+2}$ and o.4 mM each dNTP with or without dye. (KAPA BIOSYSTEMS).

A ladder (KAPA BIOSYSTEMS) size of amplicons KAPA Universal ladder contain (100 ng/µl) 1^{x}1 ml KAPA loading dye (60x) ^{x}1.5 ml contain eighteen DAN segment in bp (100, 150, 200, 300, 400, 500, 600, 800, 1000. 1200, 1600, 2000, 3000, 4000, 5000, 6000, 8000, 10000 for the orientation.

Sau gene to specific species S aureus.PCR amplification was don as follow 5 µl of DNA was added to 20 µl of **master mix** with 0.6 µmol each primers for *Sau* gene to specific species *S. aureus* described by Ruzickova*et al.*[20] The PCR cycling done were an initial denaturation step (94 C° 4min) 25 cycle amplification (denaturation 94 C° 30 s) (annealing 54 C°, 30 s) (final extension at 72 C° for 7 min) 10 µl volumes of PCR products were electrophoreses in 1.5% (w/v) agarose gel 1h ethidium bromide (0.5 µg/ml TAE) DNA were visualized in UV transilluminator.

Clumping factor *(ctfA*) gene, this genes encoding clumping factor *(ctfA)* (94 C° initial denaturation at for 4 min), 38 cycle (94 C° for 60s denaturation, 58 C° for 60s annealing, 72C° for 60s extension) and final extension at 72 C for 5 min.[21]

IgG binding –region of protein A *(spa)* gene, the PCR amplification was don under 94 C° for 2 min was an initial denaturation , 30 cycle (94 C° for 3 min denaturation , 58 C° for 30s annealing ,72 C° for 45s extension) and 72 C° for 10 min final extension. The sequences of the oligonucleotide primers summarized in Table 1.

Thermonuclease (nuc). Reaction mixtures (25 µl) included 2 µl template DNA, 20µl of master max**,** 10 pmol of each of the 2 primers 279 bp. Amplification and primer described by Stepan et al.[21] PCR cycles 37 denaturation (94°C for 1 min), annealing (55°C for 0.5 min), and extension (72°C for 1.5 min), final extension (72°C for 3.5 min). Amplified products were separated by agarose gel (1.7% agarose) used for electrophoresis at 5 V/cm for 3 h.

Table 1: Primers for amplification of the *S. aureus* gene (gene sequence (5-3) size of amplified bp

Gene	Sequence	Size bp	Reference
Sau	F:ATAAGAGATGGCGGTACTAAA R:TAAGGCGGATTACACGTTACT	530	(Ruzickova et al., 2005)
CtfA	F:GGCTTCAGTGCTTGTAGG R:TTTTCAGGGTCAATATAAGC	980	(Stepan et al., 2001)
Nuc	F:CGATTGATGGTGATACGGTT R:ACGCAAGCCTTGACGAACTAAAGC	279	(Stepan et al., 2001)
Spa IgG Binding	F:CACCTGCTGCAAATGCTGCG R:GGCTTGTTGTTGTCTTCCTC	950	(Stepan et al., 2001)
Mec A	F:5' AAA ATC GAT GGT AAA GGT TGG C 3' R: 5' AGT TCT GCA GTA CCG GAT TTG C 3'	533	(Murakami et al. 1991)

Cefoxitin sensitivity testing

This test was done according. [22]Kirby and Bauer, (1966), by disc-diffusion method, staphylococcal isolates were tested for their sensitivity to Cefoxitin (cx 30 mg). A zone of inhibition with a diameter of ≤ 21 mm was considered as an indication for resistance to methicillin. Out of the 64 samples enrolled in the current study therewere only 14 samples showed resistance to cefoxitin (30 mg) can be considered as methicillin resistant *Staphylococcusaureus.*

Oxacillin Disk Diffusion Test

Oxacillin disks (1 µg, Oxoid) were used was performed on all isolates of S. aureus using the Kirby-Bauer disk diffusion method. Zones of inhibition were determined in accordance with procedures of the CLSI standard guidelines. [23] According to CLSI, were . S. aureus isolates were considered susceptible to oxacillin inhibition zones more than 13 mm after incubation on 2% NaCl Mueller Hinton agar at 35C° for 18-24 h.

All isolates of *S. aureus* using the Kirby-Bauer disk diffusion method to detection antibiotic sensitivity, zones of inhibition were determined in accordance with procedures of the CLSI standard guidelines Ciprofloxacin, CIP, 5 µcg. Clindamycin, DA, 2 µcg, Cloxacillin, CX, 10 µcg. Erythromycin, E, 15 µcg. Gentamycin, CN, 10 µcg, Penicillin, P, 10 µcg. Streptomycin, S, 10 µcg. Tobramycin,

TOB, 10 µcg. Vancomycin, VA, 30 µcg, tetracycline, TE, 30 µcg.

PCR amplification for detection of mecA gene)
All S. aureus isolates resistance to cefoxitin (30 mg) were screened for mecA gene by PCR. The mecA gene was amplified using primers as described byMurakami et al. [24]For mecA gene, reaction mixtures (25 µl) included 2 µl template DNA, 20µl of master max**.** 20 pmol concentrations of forward and reverse primers. The cycling parameters were as follows: an initial denaturation at 94°C for 5 min; followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 70°C for 1min; and with a final extension at 72°C for 5 min . PCR products were visualized on 2% agarose gel with ethidium bromide dye under UV transilluminator. Amplicons of 533 bp were consistent with mecA gene amplification.

Biotyping
Biotyping was based on three properties-hydrolysisof Tween 80, pigmentation on Tween 80 Agar. Tween 80 1% v/v was incorporated in Nutrient Agar, and the test organism was spread over an areaapproximately 1 cm in diameter. Plates were incubatedat 37°C for 2-3 days. A positive test was denoted by a halo of fatty acids around the inoculum.This medium enhanced pigmentation of *S. aureus* andisolates were described as gold, buff or cream.

Virulence factors characterization *S.aureus* virulence factors were phenotypically characterized *in vitro*. Biotypes A B, C and D were identified by culturing isolations in brain and heart agar with violet cristal (1:10000) at 37°C. Positive colonies were determined for presenting violet color. Different biotypes were considered according to color and growth. Biotype A growth and positive to violet crystal, biotype B white, biotype C yellowish and no growth for biotype D.[25.]

3. Statistical Analysis

S. aureus frequency isolation comparison, virulence factors expression and frequency of association, was determined by $\chi 2$ test based on a cut point for positive and negative results. Statistical significance was established at a 5% level using the statistical SSPS system

4. Results

In this study was collected 130 sample of bovine mastitis and 130 from human worker in bovine farm, in bovine mastitis the percentage of isolation was appeared (47.69%) and the most strain of isolation biotype c (82.25%) and some strain was shown biotype A and (12.90%) biotype B (4.83%).

While the percentage of human isolation was shown (44.61%), the most strain was recorded biotype A (91.37%) and (8.6%) was appeared biotype C without recorded any strain type B this results depended on growth on crystal violate and hydrolysis of tween 80 and pigment productiontable.1.

Biotypes	A	%	B	%	C	%	NO	%
Bovine strain	8	12.90	3	4.83	51	82.25	62/ 130	47.69
Human strain	53	91.37	0	0	5	8.6	58/ 130	44.61
Growth on crystal violate	Violate		White		Yellow			
Hydrolysis of tween 80	-		-		+			
Pigment production	Cream		buff		variable			

Figure 1: The percentage of *S. aureus* isolation and biotypes differentiation in bovine and human.

All isolation strain was testing in the some virulence factor, all strain can detection by used *Sua* gene that specific spices detection of *S aureus*. When study of *ctf A* gene the percentage was appeared 69.35% in bovine strain and 72.41% in human strain, while the *spa* gene was shown 62.35% in 950bp and 4.68% in 390bp in bovine strain and 77.58% in 950bp and 8.62% in human strain, *nuc* gene was shown 87.93% in bovine strain while 87.93% in human strain with significant differences between virulence factor. After the test of resistance to methicillin *mec A* gene the bovine strain was shown 28.12%, while the 35.48% in human strain Table. 2

Table 2: Number and percentage of some virulence genes in the *S aureus*.

No.	*Sau* gene	*ctfA* gene	*spa* gene		*Nuc gene*	*Mec A*
Bovine	530 bp	985 bp	950 bp	390 bp	278 bp	533bp
62	62	43	40	3	53	18
Percentage	100%	69.35%	62.5%	4.68%	82.81%	28.12%
Human 58	58	42	45	5	51	22
Percentage	100%	72.41%	77.58%	8.62%	87.93%	35.48%

The all strain test to antibiotic sensitivity to Cefoxitin (cx 30 mg) and Oxacillin disks (1 µg,) the strain of MRSA was shown high sensitivity to cefoxitin comparison to oxacillin, the number of strain resistance to cefoxitin was appeared carry the *mce A* gene when used PCR technique, table 3.

The all isolation strain was test by antibiotic sensitivity by used 10 different antibiotic he high percentage of resistane to Cefoxitin, Cloxacillin, Oxacillin, Penicillin fallowed by Streptomycin and Erythromycin and the high sensitivity was shown in Vancomycin and Clindamycin in both bovine and human strain table 4, 5. With highly significant differences in p>0.00.1. The multidrug resistance more than 9 antibiotic of bovine strain (50% of MRSA) while in human strain the multidrug resistance was appeared in (40.90), Table 6.7.

Table 3: The sensitivity of Oxacillin and cefoxitin comparison with PCR

Strain	OX disk	False positive	Cef disk	False positive	PCR mec A	False positive	True MRSA
Bovine 62	23	5	18	0	18	0	18
Sensitivity rate	21.73%		0%		0%		29.03%
Human 58	33	11	23	1	22	0	22
Sensitivity rate	33.33%		4.34%		0%		37.93%

Table 4: The sensitivity and resistance of different antibiotic in bovine strain

Antibiotic	*Sensitive*	*Percentage*	*Resistance*	*Percentage*
Cefoxitin, Cloxacillin, Oxacillin, Penicillin	0	0%	22	100%
Streptomycin	6	27.27	16	72.73%
Erythromycin	7	31.81%	15	68.19%
Gentamycin	7	31.81%	15	68.19%
Tetracycline	7	31.81%	51	68.19%
Ciprofloxacin	9	40.90%	13	59.10%
Tobramycin	14	63.63%	8	36.37%
Clindamycin	14	63.63%	8	36.37%
Vancomycin	15	68.19%	7	31.81%
X2= 35.552		P= 0.00021		

Table 5: The sensitivity and resistance of different antibiotic in human strain

Antibiotic	Sensitive	Percentage	Resistance	Percentage
Cefoxitin, Cloxacillin, Oxacillin, Penicillin	0	0%	18	100%
Streptomycin	4	22.22%	14	77.88%
Erythromycin	4	22.22%	14	77.88%
Gentamycin	4	22.22%	14	77.88%
Tetracycline	3	16.66%	15	83.44%
Ciprofloxacin	9	50%	9	50%
Tobramycin	13	72.22%	5	27.88%
Clindamycin	11	61.11%	7	38.99%
Vancomycin	13	72.22%	5	27.88%
X2 =43.438		P= 0.0001		

Table 6: The multidrug resistance of different antibiotic in human strain

Resistance to antibiotic	Number of strain	Percentage
Nine antibiotic or more above	9	50%
Eight antibiotic	2	11.11%
Sven antibiotic	4	22.22%
Six antibiotic	1	5.55
Five antibiotic	2	11.11
	18	100%
	X2 =42.800	P=0.0003

Table 7: The multidrug resistance of different antibiotic in bovine strain

Resistance to antibiotic	Number of strain	Percentage
Nine antibiotic or more above	9	40.90%
Eight antibiotic	3	13.63%
Sven antibiotic	1	4.54%
Six antibiotic	5	22.72
Five antibiotic	4	18.18
	22	100%
	X2 =48.000	P= 0.0002

5. Discussion

Mastitis is the most common cause for antibiotic use in dairy herds. Effectiveness of current treatments and ability to control infectious diseases in both animals and humans may become hazardous.

In general, variation in the prevalence of genes between these studies might be attributable to different factors such as the geographical difference, type of clinical sample, number of samples, specificity and sensitivity of methodology and types of strains (MRSA or MSSA).

In this study was determine methicillin resistant staphylococci a zoonotic importance associated with mastitis in cattle and their role in transmission to animal handlers.

Our study confirms the usefulness of PCR for the detection of antibiotic resistance genes associated with *S. aureus* infections. The PCR offers a rapid, simple, and accurate identification of antibiotic resistance profiles.Laboratory methods used to detect multidrug resistant bacteria such as MRSA should have high sensitivity and specificity.[26]

Some study combined in same line,a total of 151(31.45%) *S.aureus* isolates were identified by API-Staph® detection, (41.05%) isolates were determined as resistant to Cephoxitin (30 ig) demonstrates the distribution of *mecA* carrying *S. aureus* isolates and their locations in Turkey.[27]

Other study reviled the results combined with this study, A total of 235 clinical mastitis milk samples revealed 116, (49.36%) samples positive for *S. aureus,* of which 12 samples (10.34%) were MRSA.[28]

However, improper use of antibiotics creates problems such as the emergence of bacterial resistance to antibiotics.[6]

One of these problems, the occurrence of methicillin resistance, has been observed frequently in recent years.[3]

Therefore, infections by MRSA require rapid and accurate diagnosis for elimination at an early stage, because these strains can cause severe damage to infected sites and may be widespread in the environment.[29]

In most routine microbiological settings, the detection of methicillin resistance among staphylococcal isolates is based on phenotypic assays such as the disk diffusion test and MIC determination. Genetic confirmation of positive findings based on detection of the *mecA* gene has also been reported.[30]

MRSA strains were multi-drug resistant which might be due to production of betalactamase and PBP2a (penicillin binding protein).[31]

The disk diffusion and broth dilution method require atleast 24 h for evaluation of the results. However, thedetection of antibiotic resistance genes such as *mecA*gene by PCR techniques is considered the gold standard method.[32]

A goodcorrelation between phenotypic antibiotic susceptibility patterns and genotypic analysis by PCR wasalso reported.[33, 34, 35]The critical para meters for success of a PCRfor the detection of multidrug resistant bacteria asMRSA are cost, reliability and practical, fast,accuracy and sensitivity and results were obtainedwithin 4 hours.[32] in veterinary microbiologynumerate techniques applicable to diagnosisused tocharacterize*S. aureus*strainsthat important cause of bovine mastitis and Rapid detection necessary to

detection of epidemiologicaland therapeutic purposes when treated MRAS.[36]

TNase producing by *S. aureus* encoding by*nuc* genethese bacteria. *S. aureus* TNase has of the *nuc* genehas potential for the rapid diagnosis of *S. aureus* infections with contain Species-specific sequences and amplification.[36, 37]

The tests of susceptibility antimicrobial are provide guide to the veterinarian to help in selecting treatment of mastitis that caused by *S. aureus*.[38] Many studies are available to recorded MRSA and multi-resistant drug of *S. aureus* strains and has also beenreported in some cases in veterinary medicine.[9, 39]

Differencesstrain genetic variability and phenotypical expression of virulence factors related to severity of clinical signs in *S. aureus* mastitis.[40]

The host specificity of *S. aureus* strains of diverse origin related with analyses of the genotype distributions of demonstrated. It seems that the occurrence of some *S. aureus* strains lineages is restricted to animals.[41,42]

In the studyof Vishnupriyaet al .[26]that collect 158 samples from mastitic milk and 126 nasal swabs from the animal handlers when analysis of phenotypic and genotypic six MRSA isolates (three bovine and three human)Control of mastitis staphylococci may occurs by control to milk from harbor organisms that causes by potentially pathogenic to humansbecause this bacteria most commonly isolated from subclinical mastitis.[43, 44]*S. aureus* strains produce penicillinase and more than 80% of thus strain carry β-lactam and resistance to different antibioticssuch as methicillin, (MRSA) had become a serious nosocomial infection worldwide.[45]

MRSA isolation from bovine mastitis so milk could be a source of MRSA infection to human beings. Prevalence of MRSA among the two groups were found to be 8/19 (42.1%) and 2/21 (9.5%) respectively healthy medical students and healthy nursing students.[46]No report that recoded to systematic study explain relatedness of MRSA between animals and humans and vice-versa.[47]

Different animals such horses and dogs with lesions, and dogs and cats that were carriers MRSA strains and isolated from cows with mastitis.[48] Transmission of MRSA between humans and animals (e.g., dogs, horses, pigs) has been reported.[48, 49, 50]

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