Journal homepage:
http://www.bsu.edu.eg/bsujournals/JVMR.aspx

Online ISSN: 2357-0520 Print ISSN: 2357-0512

Original Research Article

Vancomycin resistance among methicillin-resistant *Staphylococcus aureus* isolates from animal milk

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ABSTRACT

Staphylococcus aureus(*S. aureus*) is a major cause of mastitis in dairy animals and a serious pathogen affecting human health. The current study was designed to investigate the extent of *S.aureus* in milk samples collected from dairy animals as well as human clinical samples,beside determination of its antimicrobial susceptibility pattern. Also, the prevalence of both *mec*A and *van*A genes among some selected methicillin-resistant isolates was investigated. Out of 120 milk samples obtained from different animals (cows, buffaloes, sheep, and goats), 81 (67.5%) samples reacted positive for *S. aureus*, whereas 67 (74%) out of 90 human samples were found positive for *S. aureus*. Disk diffusion susceptibility testing revealed that *S.aureus* isolates of humans were more resistant than thoseof animals against all tested antimicrobials except for clindamycin. A high rate of multi-drug resistance (MDR) and *mec*A gene was recorded in *S. aureus*of both animals and humans. Surprisingly,*van*Agene, which is responsible for vancomycin resistance was detected only in *S. aureus* isolated from animal milk. To the best of ourknowledge, it is the first record of *van*A gene in *S. aureus* recovered from animals.

ARTICLE INFO

Article history:

Received 10/2017

Accepted 11/2017

Online 11/2017

Keywords:

Vancomycin,

Staphylococcus aureus,

Animal milk

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1. Introduction

Staphylococcusaureus(*S.aureus*)is considered as one of the bacterial commensals, however it may act as a virulent pathogen threatening both animals and humans (Thammavongsa et al., 2015,Carfora et al., 2016; Li et al., 2017).*S.aureus*isfrequently reported as a cause of mastitis in dairy animals (Aires-de-Sousa et al., 2007).As well asit causes a variety of hospital and community acquired clinical infections in humans, including skin, soft tissue, andpleuropulmonary infections(Tong et al., 2015).

The ability of *S. aureus* to outwit the immune system, above and beyond its multi-drug resistance (MDR) phenotype makes it as one of the most intractable pathogenic bacteria in the history of antibiotic

chemotherapy(Hiramatsu et al., 2014).Methicillin and vancomycin resistanceare the two most notablepatterns. The spread ofmethicillin-resistant *S. aureus*(MRSA) has become a significant concern for both animal and human health worldwide (Ba et al., 2014; García-Álvarez et al., 2011;van Rijen et al., 2014).Methicillin resistance in *S. aureus* is predominantly mediated by the expression of *mec*A gene, which is located on a mobile genetic element; the staphylococcal cassette chromosome mec (SCCmec), encoding an altered penicillin-binding protein (PBP2a) with an exceedingly low susceptibility to beta-lactam antibiotics.Thus,*S. aureus*will bepractically resistanttomost beta-lactam antibiotics(Hiramatsuet al., 2001).On the other hand, resistanceto vancomycinisaccomplished by horizontal transfer of a plasmid-born transposon carrying*van*Agene from vancomycin-resistant *Enterococcus* to *S. aureus*crosswise the genus barrier(Hiramatsu et al., 2014).

Hence,the current study was designed to investigate the presence of *S. aureus* in milk samples collected from dairy animals as well as human clinical samples, beside determination of its antimicrobial susceptibility pattern. Also, the prevalence of both *mec*A and *van*A genes among some selected methicillin-resistant isolates was investigated.

2. Materials and methods

Sample collection

The samples were obtained fromEl-Minia Governorate, Egypt,situated 241 km south to Cairo, Egypt.

Milk Samples

One hundred and twenty milk samples were collected fromdairy animals including cows, buffaloes, sheep, and goats (30 samples of each). The samples were obtained from animals suffering from clinical and subclinical mastitis and transferred to the laboratory in an ice-cooled container

Human samples

Ninety clinical swabs were collected from abscesses, wounds, nose, and ears of patients attending various departments inEl-Minia University hospital. An oral approval was taken from the individuals included in this study before collection. Both milk samples and swabs were transferred to laboratory inan ice-cooled container for processing.

Isolation and identification of S. aureus

An aliquot (10 µl) from each milk sample was inoculated onto mannitol salt agar and incubated at 37^{o}C for 24h. On the other hand, the swabs were inoculated overnight into trypticsoya broth to be further cultivated onto mannitol salt agar and incubated at 37^{o}C for 24h.Isolated yellow colonies showedGram and catalase positive cocci were further identified according toCollee et al.(1996).

Antimicrobial susceptibility of S. aureus isolates

Susceptibility of *S. aureus* isolates to the antimicrobial agents was tested using disk diffusion method according to the Clinical and Laboratory Standards Institute(CLSI, 2014). Eight antimicrobial agents of both veterinary and public health significance were used;Imipenem (IMP 10µg), ciprofloxacin(CIP 5µg), amoxicillin/clavulanic acid (AMC 20/10µg),cefotaxime (CTX30µg), clindamycin(DA 2µg),cefoxitin(FOX30µg),sulphamethoxazole/trimethoprim(SXT 1.25/23.75µg),and doxycycline (DO 30µg).Resistance to cefoxitin was used as an indicator of methicillin resistance(CLSI, 2014).

VancomycinSusceptibility testing

Resistance to vancomycin disk (30-µg) was determined to detect isolates containing the *van*Avancomycin resistance gene (VRSA)using disk diffusion method.Such isolates must show no zone of inhibition around the disk (zone=6 mm) following the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2012).

Genomic DNA extraction

Genomic DNAs of *S. aureus*isolates were extracted using a Thermo Scientific Gene JET Genomic DNA Purification Kit, sigma, USAaccording to the manufacturer's protocol.

PCR detection of mecA and vanA genes

The primers used for amplification of

*mec*A and *van*A genes are listed with their sequence and references in table (1).

Amplification of mecA gene

PCR was performed with initial denaturation step at 95°C for 5 min, followed by 40 cycles of amplification consisting of 1 min of denaturation at 95°C, annealing at 47°C for 30 s and extension at 72°C for 30 s with a final extension step at 72°C for 5 min.

Amplification of vanA gene

PCR was performed with initial denaturation step at 94°C for 3 min, followed by 30 cycles of amplification consisting of 1 min of denaturation at 94°C, annealing at 54°C for 1 min and extension at 72°C for 1min with a final extension step at 72°C for 7 min.

Table 1. Types of genes, primers sequence and references

Target gene	Primer Design	Product size (bp)	Reference
*mec*A	F: 5' TAG AAA TGA CTG AAC GTC CG 3' R: 3' TTG CGA TCA ATG TTA CCG TAG 5'	154	(Schuenck et al., 2006)
van A	F: 5' GGGAAAACGACAATTGC3' R: 3'GTACAATGCGGCCGTTA5'	732	(Depardieu et al.,2004)

3. Results

Isolation rate of S.aureus

Examination of milk samples from different animals revealed that *S. aureus* was recovered at a rate of 67.5% (81 out of 120 samples). The highest rate of isolation was from cow's milk (70%), while the other animals exhibited an equal rate of recovery (66.6%). Regarding humans, 74% (67 out of 90 samples) were found positive for *S. aureus.*

Antimicrobial susceptibility of S.aureus isolates

Disk diffusion susceptibility testing revealed that human isolates exhibited a higher resistance than animal ones against all the tested antimicrobials except for clindamycin. Concerning imipenem, the results indicated that all animal isolates were found susceptible for it (Table 2).Additionally, MDR (i.e. resistance to three or more antimicrobials of different tested classes) was recorded at a higher percentage among*S. aureus* recovered from humans (70%) than that of animals (34.6%).

Phenotypic characterization of methicillin-resistant S. aureus among the tested isolates

A total of 56 (83.6%) and 49 (60.5%) isolates of human and animal origin respectively showed resistance against cefoxitin and consequentlywere categorized phenotypically as MRSA (Table 2).

Detection of mecA and vanA genes among MRSA

A total of 25 MRSA isolates (5 isolates from each host species) were investigated for detection of *mec*A and *van*A genes. These isolates were MDR, resistant tocefoxitin and showed no inhibition zone around vancomycin disk.

The highest prevalence for *mec*A gene was detected amongst the isolates of cows, buffaloes, and humans (80% each). However only one (20%) isolate obtained from sheep was found positive. On the contrary, none of the goat'sfound harboring this gene (Table 3).

Regarding *van*Agene, the highest prevalence was noticed among *S. aureus* isolated from sheep (100%) while isolates recovered from cows, buffalo's and goat's milk revealeda prevalence rate of 40% for each host species. On the opposite side, all the tested human samples were negative for *van*A gene.

In general, both *mec*A and *van*A genes were coexisted in 5 isolates out of 25 tested ones (2, 2, and 1 from cows, buffaloes and sheep respectively). On the contrary, both genes were absent in 6 isolates (3 from goats and 1 from

each of cows, buffaloes, and sheep). Fig (1) showed example of some isolates that harbored the expected band of *van*A genes.

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Table 2.Percentage of antimicrobial resistant *S.aureus*of both animals and humans origin

Antimicrobial Agent	Cows isolates No(%*)	Buffaloes isolates No (%)	Sheep isolates No (%)	Goats isolates No (%)	Total animal isolates No (%)	Humans isolates No (%)
Imipenem	0 (0%)	0(0%)	0(0%)	0(0%)	0(0%)	18 (27.0)
Cefoxitin	14(66.7)	8(40.0)	11(55.0)	16(80.0)	49(60.5)	56(84.0)
Cefotaxime	4(19.0)	4(20.0)	0(0%)	10(50.0)	18(22.2)	55(82.1)
Amoxicillin/ clavulanic acid	5(23.8)	7(35.0)	12 60.0)	17(85.0)	41(50.6)	56(84.0)
Trimethoprim\ sulfamethoxazole	12(60.0)	3(15.0)	7(35.0)	11(55.0)	33(40.7)	54(80.6)
Ciprofloxacin	3(14.3)	2(10.0)	1(5.0)	0(0%)	6(7.4)	50(74.6)
Clindamycin	17(81.0)	18(90.0)	14(70.0)	19(95.0)	68 (84.0)	59(88.1)
Doxycycline	9(42.9)	5(25)	1(5)	4 (20)	19(23.5)	49(73.1)
MDR	9(42.9)	10(50)	6(30)	12 (60)	37(45.7)	40 (59.7)

%: means percentage of resistant isolates in relation to the total tested isolates

Table 3. Distribution of *mec*A and*van*A genes among the selected *S.aureus* isolates

Species	Number of examined isolates	*mec*A positive		*van*A positive	
		Number	%	Number	%
Cows	5	4	80	2	40
Buffaloes	5	4	80	2	40
Goats	5	0	0	2	40
Sheep	5	1	20	5	100
Humans	5	4	80	0	0
Total	25	13	52	11	44

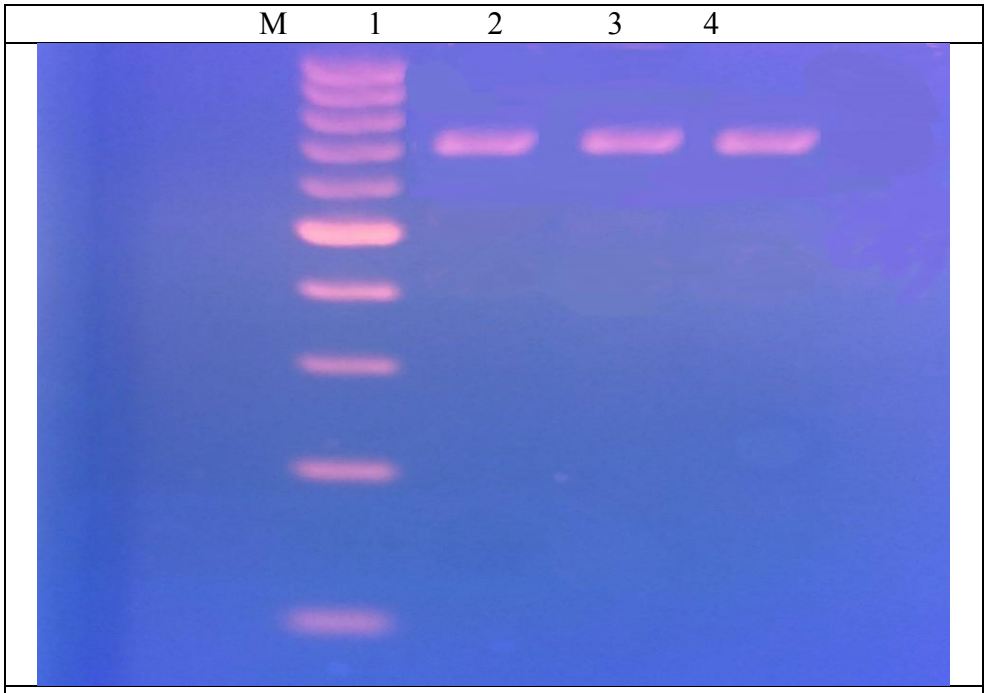

Fig 1: Agarose gel electrophoresis of PCR products stained with ethidium bromide. *van*A gene (732bp),
M: 100 bp plus ladder (Size range: 100-1000 bp)
positive samples: lane 1, 2, 3
negative control: lane 4

4. Discussion

In the currentstudy,*S. aureus* was detected in 67.5% of the examined milk samples. There wasn't notable difference in the prevalence in relation to the animal host. Theobtainableprevalence is much higherthan that previously reported byElhaig and Selim(2015)in Egypt (40%) andLi*et al.*(2015) in China (56.5%),however,a higher rate(90%) was obtained in a study carried on goats with subclinical mastitis in Brazil (Martins et al., 2015).On the other hand, a prevalence of 74% was recorded from human clinical samples whichisnearer to previous studies in Egypt (Sobhy et al., 2012; Ahmed et al., 2014).Thehigh isolation rates in the present work reflect the predominance of *S.aureus*in both animal milk and human clinical samples in the investigated area.

Testing the antimicrobial susceptibility of the recovered isolatesagainst drugs of both veterinary and human medicine exhibited worrisome findings. Alongside the high rate of methicillin resistance and MDR, vancomycin resistance was confirmed in*S. aureus* isolates of animals. The multiple resistance attitudes must be of concern since antibiotic resistance is carried on plasmids that can be transferred from one staphylococcal species to another (Werckenthi et al., 2001). Several reports worldwide have described MDR among *S. aureus* of both human and animal sources(Kumar et al., 2010; Shi et al., 2010; Hiramatsu et al., 2014).

MRSA was first appeared in the 1960s, soon after methicillin was introduced into clinical therapeutics.Thereafter, it was responsiblefor hospital outbreaks in Western Europe, Australia, and the United States (Barber, 1961;Jevons and Parker, 1964). Phenotypically, the present work revealed a total of 56 (83.6%) and 49 (60.5%) isolates of humans and animals respectively as MRSA.In Egypt, it was recorded

that the prevalence was as high as 52% during the period between 2003 and 2005 (Falagas et al., 2013).

A total of 25 MRSA isolates were selected for the detection of *mec*Agene. The highest prevalence of *mec*A gene was detected among the selected isolates of cows, buffaloes and humans (80% each).Although all the tested isolates were phenotypically positive MRSA, 13 (52%) harbored *mec*A gene. This phenomenon coincided with that obtained by several literatures (Turutoglu et al., 2009; Kumar et al., 2010; Li et al., 2015).This might be attributed to the existence of *mec*A homologue known as*mec*C(García-Álvarez et al., 2011)or due to the occurrence of some mutations in the penicillin-binding protein genes (Turutoglu et al.,2010; Ba et al., 2014).

For long periods,vancomycin was considered as the drug of choice for treating *S.aureus*infections, particularly MRSA (Holmes et al., 2012). However,a reduced susceptibility against it was observed for the first time in Japan(Hiramatsu et al., 1997).Vancomycin-resistant*S. aureus* (VRSA) strains whose resistance is due to acquisition of *van*A resistance gene from enterococci was first emerged in the United Statesin 2002. Later, VRSA wasdetected in Iran and India, even though it remains rare cases worldwide (Howden et al., 2010; Holmes et al., 2012).A previousliterature in Egypt has recorded resistance against vancomycin in *S.aureus* of animal origin, but it was not accompanied by genetic characterization (Radwan et al., 2015).In the current study, the same MRSA isolates (*n*=25) which were selected for the detection of *mec*A gene were simultaneously investigated for *van*A gene.The highest prevalence among the tested isolates was noticed in *S.aureus* isolated from sheep (100%), while those originated from cows, buffalos, and goats revealed a prevalence of 40% each. On the contrary, all tested isolatesrecovered from human samples were negative for *van*Agene.The emergence of resistance against vancomycin, not commonly used in livestock, could be as referred to the fact that livestock act as a reservoir of vancomycin-resistant *Enterococcusfaecalis*possessed the *van* Agene(Bates et al., 1994).Furthermore,*S. aureus*has the ability to secrete an *E. faecalis*-specific sex pheromone that trigger genes transfer, including vancomcyin resistance gene, which was previously proved in the laboratory byNoble et al. (1992).

This study concluded the predominance of *S. aureus*in human clinical samples as well as milk samples of dairy animalssufferedfromeither clinical or subclinical mastitis,a high rate of methicillin resistance, and MDR. Vancomycin resistance was confirmedby detection of *van*A gene in *S. aureus* of animals. Consequently, effective measures are needed to identify causes of emerging vancomycin-resistant *S. aureus* in animals toavoid the potential of its transfer to humans.

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