Journal of Applied Veterinary Sciences, 9 (3): 1-10 (July, 2024).
ISSN: Online: 2090-3308, Print: 1687-4072
Journal homepage : https://javs.journals.ekb.eg

Detection of *clf*A, *clf*B and *coa* genes in Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolated from Nasal Cavity of Cows, Buffalo and their Breeders in Nineveh Governorate, Iraq

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ABSTRACT

The present study aimed to isolate and identify Methicillin-Resistant DOI*Staphylococcus aureus* MRSA from the nasal cavity of healthy cows and buffaloes and their breeders in Nineveh Governorate and detect some virulence factors by using molecular methods. A total of 150 samples of cotton swabs were collected randomly from different areas of Nineveh governorate. The samples were cotton swabs from the nasal passages of healthy cattle, buffaloes, and their breeders (50 swabs of each type). All the samples were subjected to culture and molecular testing. The results showed the highest isolation percentage of *S. aureus* from cattle followed by breeders, then buffaloes, at 54%, 40%, and 32%, respectively. The total isolation percentage of MRSA was 65.1%. The highest percentage was in buffaloes, followed by breeders and cattle, at 93.75%, 70%, and 44.44%, respectively. Out of 41 isolates from cattle, buffaloes, and their breeders, the virulence genes *clf*A, *clf*B, and *coa* were detected in MRSA at rates of 100%, 80.49%, and 65.85%, respectively. The current study concluded that cattle and buffalo are considered carriers and potential transmitters of MRSA, which makes them risk factors for infection in humans, especially those who are in direct contact with animals. Together, these findings also highlight the need to prevent the transmission of zoonotic pathogens to humans via occupational exposure or consumption of contaminated animal products.

Original Article:
DOI:https://dx.doi.org/10.21608/javs.2024.276537.1324

Received : 12 March, 2024.
Accepted: 06 May, 2024.
Published in July, 2024.

Keywords: MRSA, Nasal cavity, Nineveh Governorate, *S. aureus*, Virulence gene.

J. Appl. Vet. Sci., 9 (3): 1 - 10.

INTRODUCTION

Staphylococcus aureus is considered one of the major bacteria in its genus out of more than 50 identified species. It is a Gram-positive, facultative, anaerobic, catalase-positive, and salt-tolerant (NaCl) bacterium (**Gnanamani *et al.*, 2017; Rossi *et al.*, 2020**). It tolerates temperatures ranging from 7 to 40°C and has the ability to survive in unsuitable conditions, which makes it a persistent and widespread pathogen in the environment (**Gnanamani *et al.*, 2017**). It is naturally found on the mucous membranes and skin of animals and humans, and its rate of presence varies depending on the type of host (**Gnanamani *et al.*, 2017**).

Staphylococcus aureus has the ability to attach to a wide spectrum of host cells and reproduce inside the host cells (**Watkins and Unnikrishnan 2020**). It colonizes the body's skin and mucous membranes, such as the nostrils, which are the most colonizing sites, which increases the risk of infection (**Chen *et al.*, 2017; Sakr *et al.*, 2018**). *S. aureus* is one of the most common and life-threatening pathogens in farm animals and pets. It causes various forms of infection, ranging from skin and soft tissue infections to the lower respiratory tract and serious deep infections such as endocarditis, mastitis, bone myelitis, sepsis, and necrosis, as well as food contamination (**Furuya and Lowy, 2006; Kotb and Gafer, 2020**; **Kwiecinski and Horswill, 2020; Fikry *et al.*, 2021**). Its incidence is increasing in both humans and animals, affecting public health and causing significant economic burdens (**Scherrer *et al.*, 2004; Veras *et al.*, 2008; Organji *et al.*, 2018; Mubarak, 2021; Zedan *et al.*, 2022**).

Among the identified and most pathogenic *S. aureus* pathogens is MRSA, which has been described by the World Health Organization (WHO) as a priority pathogen (**Stapleton and Taylor, 2002; Shrivastava *et al.*, 2018**). It is characterized by its possession of the *mec*A gene, which is the main gene responsible for methicillin resistance. This gene was found in a genetic location called the staphylococcal cassette chromosome *mec* (SCC*mec*) (**Fishovitz *et al.*, 2014),** which was discovered in *S. aureus* and isolated from animal and environmental samples (**Paterson *et al.*, 2014; MacFadyen *et al.*, 2018**).

The degree of *S. aureus* pathogenicity depends on several surface components and extracellular proteins. The expression of most virulence factors in *S. aureus* is controlled by the Accessory Gene Regulator locus (AGR), which encodes a two-component signaling pathway that forms the activating ligand and is a bacterial density sensor peptide called "autoinducer peptide," which is also encoded by AGR (**Jenul and Horswill, 2019**). Thus, the AGR system is directly involved in controlling the expression of virulence factors in *S. aureus* (**Oliveira *et al.*, 2018**).

The evolution tracking studies of MRSA showed that bovine strains (LA-MRSA) lost host specificity and were easily transmitted from animals to humans and vice versa. In addition to the fact that raising cows and buffalo has increased its spread in both directions, this has led to the transmission of some types of MRSA from human origin to cows and buffalo and vice versa, leading to their evolution and adaptation through the loss of unbenefited virulence factors in the new host and the development of new transmittable genetic factors (**Richardson, 2019**). MRSA possesses genes encoding virulence factors including *hla*, *hlb*, *clf*A, *clf*B, *coa*, and *fnbp*A (**Moreno-Grúa *et al.*, 2018; Wang *et al.*, 2019**). At the same time, it also shows a multidrug resistance pattern to cefoxitin, penicillin, tetracycline, erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin, and fusidic acid linked to the *mecA* gene.

The acquisition of antimicrobial resistance effectively represents a major challenge to the medical world, both in human and veterinary terms, with regard to the treatment and control of MRSA and *S. aureus*. In order to continue and expand the continuous monitoring of endemic MRSA in humans and animals, the current study aimed to isolate and diagnose MRSA from the nasal tract of healthy cows and buffaloes and their breeders in Nineveh Governorate and to detect some virulence factors by using molecular techniques.

MATERIALS AND METHODS

This study was conducted in Nineveh Governorate, located in the northwestern part of Iraq, which is characterized by climatic conditions that vary according to its surface topography. Temperatures range between 0 and 8°C in winter and 40 and 50°C in summer. It’s characterized by a great diversity of livestock and many types of animals. Domesticated animals, such as sheep, goats, cows, and buffalo, are abundant in areas rich in fertile pastures. It's primarily an agricultural area, and the animal products constitute the second half of agricultural production.

Sampling and sample collection

One hundred and fifty cotton swabs were collected randomly from different areas of Nineveh governorate from the nasal passages of healthy cattle, buffaloes, and their breeders (50 swabs of each type) during the period from January 2023 till February 2024. The samples were collected by sterile cotton swabs placed in sterile tubes containing peptone water, then transferred directly to the laboratory of scientific research at the College of Veterinary Medicine, University of Mosul, for bacteriological examinations.

Isolation of *S. aureus*

Traditional microbiological techniques

All the samples were subjected to microbiological tests, including culturing on the selective medium mannitol salt agar (MSA) (Himedia/India), gram staining and cultivation on 5% sheep blood agar was used to test hemolytic activity of the isolates, in addition to catalase and coagulation tests (**Markey *et al.*, 2013**). CHROMagar^{TM} (Himedia/India) was used to identify methicillin-resistant Staphylococcus aureus (**Fahim *et al.*, 2023**).

Molecular techniques

Isolation of DNA

Strictly following microbiological testing, the DNA of *S. aureus* isolates was extracted and analyzed. The samples were first cultivated on Mannitol salt agar and incubated at 37°C for 24 hours. DNA was extracted from *S. aureus* isolates using the Qiagen (Germany) DNeasy Blood and Tissue Kit, according to the instructions. The concentration of extracted DNA was then measured with the Genova Nano (Jenway, UK) instrument and properly kept at -20°C.

Polymerase Chain Reaction Technique

As shown in **Table 1,** PCR technique was utilized to amplify particular sequences of the *nuc, mec*A, *clf*A, *clf*B, and *coa* genes for *S. aureus* isolates. A total of 25 µl was used for the PCR reaction mixture, which contained 12.5 µl of Promega Corporation's (2×) GoTaq Green Mix Master, 1 µl of the forward primer, 1 µl of the reverse primer, 6.5 µl of Qiagen (Germany) DNeasy-free water, and 4 µl of extracted DNA template. The entire mixture was placed in a PCR tube, and the total volume was adjusted to 25 µl. The PCR amplification was performed under specific thermal cycling conditions. These conditions, including denaturation, annealing, and extension temperatures and durations, were tailored to the PCR protocol being used and optimized for the primer set and DNA template under the study. Next, 2% agarose gel electrophoresis with Peqlab (Erlangen, Germany) was used to visualize the target sequence amplicons. A gel along with a 100-bp ladder DNA marker. Electrophoresis was carried out to separate and visualize the amplified DNA fragments, which were then compared to the DNA ladder for size estimation.

Table 1: The used primers for testing of *S. aureus*

	Gene	Primer	Primer sequence (5'-3')	Product size (bp)	PCR program	Reference
	nuc	*nuc*-F	GCGATTGATGGTGATACGGTT	279	A	(Rahman *et al.*, 2018)
		nuc-R	AGCCAAGCCTTGACGAACTAAAGC			
	*mec*A	*mec*A-F	GTGAAGATATACCAAGTGATT	147	A	(Rahman *et al.*, 2018)
		*mec*A-R	ATGCGCTATAGATTGAAAGGAT			
	*clf*A	*clf*A-F	ATTGGCGTGGCTTCAGTGCT	288	A	(Tristan *et al.*, 2003)
		*clf*A-R	CGTTTCTTCCGTAGTTGCATTTG			
	*clf*B	*clf*B-F	ACATCAGTAATAGTAGGGGCAAC	203	B	(Tristan *et al.*, 2003)
		*clf*B-R	TTCGCACTGTTTGTGTTTGCAC			
	coa	*coa*-F	ATAGAGATGCTGGTACAGG	674	A	(Javid *et al.*, 2018)
		coa-R	GCTTCCGATTGTTCGATGC			

PCR program: A: 35 times (94°C for 45s, 55°C for 60s, 72°C for 60s), B: 35 times (94°C for 45s, 60°C for 60s, 72°C for 60s

RESULTS

The results of the current study showed that the grown bacterial colonies on the MSA were yellow in colour, medium to large, round, smooth, raised, shiny, and had a buttery texture and regular edges. They were characterized by changing the colour of the agar medium from red to yellow as a result of sugar fermentation. Microscopically, they were Gram-positive and positive for catalase and coagulase. Its growth on blood agar resulted in Beta-hemolysis.

The isolation rate of *S. aureus* showed variation between traditional and molecular techniques. Out of 150 examined samples, the isolation rate using traditional techniques was 52.67%, and that using molecular techniques was 42% after electrophoresis of the amplification products for *nuc* gene on agarose gel, which were identical to the ladder size at 279 bp. **(Table 2 and Fig.1)**, the highest isolation rate by molecular techniques was recorded from cattle samples, followed by breeders, then buffaloes, at 54%, 40%, and 32%, respectively.

The total isolation percentage of MRSA among 63 *S. aureus* isolates, confirmed by the *nuc* gene was 65.1%. The highest percentage was recorded in buffaloes, followed by breeders, and then cattle, at rates of 93.75%, 70%, and 44.44%, respectively **(Table 2).**

Table 2: The examined samples, the isolation rates of *S. aureus* and MRSA by traditional and molecular techniques.

N	Sample	No.	*S. aureus*				MRSA			
			Traditional		Molecular (*nuc*)		Traditional		Molecular (*mec*A)	
			No.	%	No.	%	No.	%	No.	%
1	Cattle	50	32	64	27	54	12	44.44	12	44.44
2	Buffalo	50	20	40	16	32	15	93.75	15	93.75
3	Human	50	27	54	20	40	14	70	14	70
Total		150	79	52.67	63	42	41	65.1	41	27.33

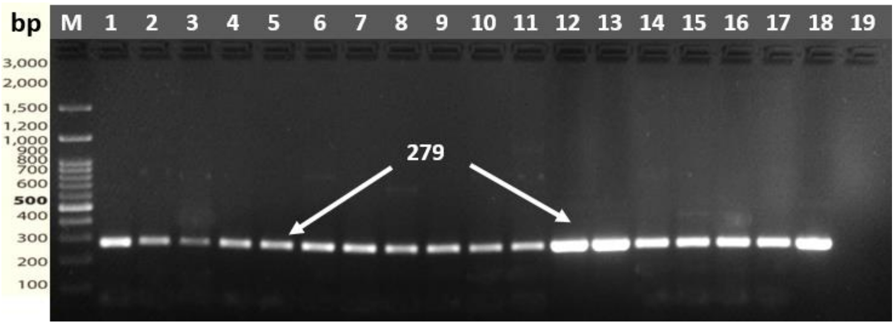

Fig. 1: PCR amplification products of *S. aureus* isolates for *nuc* gene at 279bp

The results of MRSA on chromogenic agar, showed of bluish-green colonies as shown in **Fig. 2**.

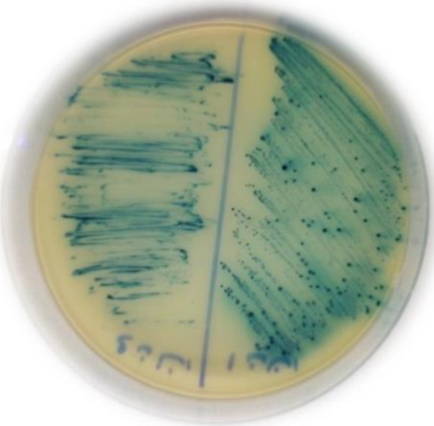

Fig. 2: The growth of MRSA on chromogenic agar

The isolation results were identical to the results of electrophoresis of amplification products for *mec*A gene at 147 bp on an agarose gel, as shown in F**ig. 3.**

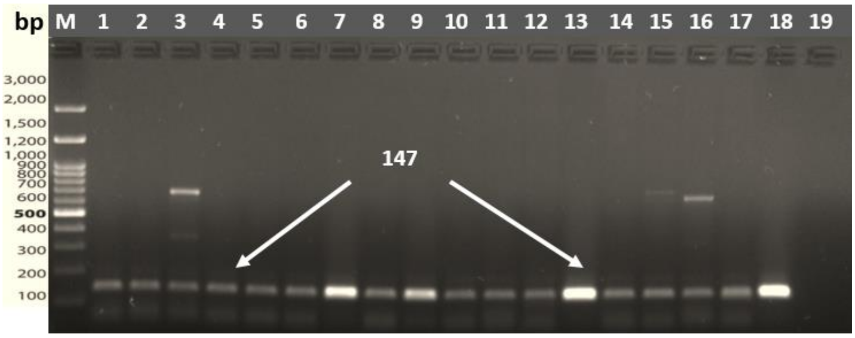

Fig.3: PCR amplification product for MRSA isolates for *mec*A gene at 147bp

Out of 41 isolates from cattle and buffalo, their breeder's virulence genes were evident in MRSA. That was evident through molecular analysis of *clf*A gene at a product size of 288 bp, 203 bp for the *clf*B gene, and 674 bp for the *coa* gene **(Figs. 4, 5, and 6)** at rates of 100%, 80.49%, and 65.9%, respectively.

The study also revealed the presence of virulence genes for MRSA, which were evident through the results of amplification and electrophoresis on agarose gel by the appearance of specific amplicon for *clfA* gene at 288 bp, 203 bp for the *clf*B gene, and 674 bp for the *coa* gene at a rate of 100%, 80.49%, and 64.28%, respectively, out of 41 isolates from cows, buffalo, and their breeders.

The *clf*A gene was detected in all the isolates (100%). The highest percentage of isolates carrying the *clf*B gene was recorded in buffaloes, followed by breeders and cattle, at a rate of 86.67%, 78.57%, and 75%, respectively. At the same time, the highest percentage of isolates carrying the *coa* gene was recorded in buffaloes, followed by breeders, then cows, at a rate of 73.33%, 64.29%, and 58.33%, respectively **(Table 3; Figs.4, 5, and 6).**

Table 3: The virulence genes recovered from MRSA isolates

Source	No.	Virulence gene					
		*clf*A		*clf*B		*coa*	
		No.	%	No.	%	No.	%
Cattle	12	12	100	9	75	7	58.33
Buffalo	15	15	100	13	86.67	11	73.33
Breeders	14	14	100	11	78.57	9	64.29
Total	41	41	100	33	80.49	27	65.9

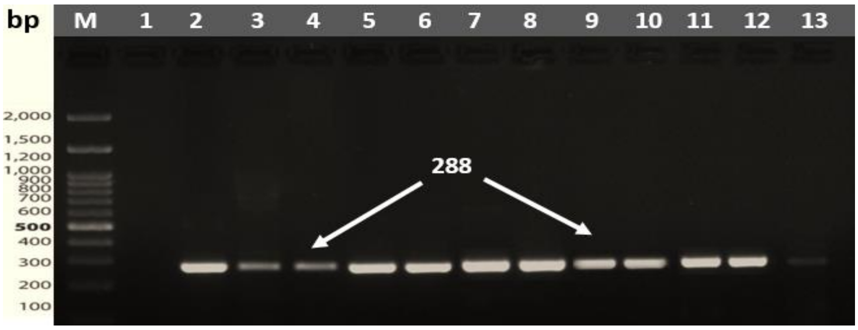

Fig. 4: PCR amplification products for *clf* A gene at product size of 288 bp for MRSA isolates

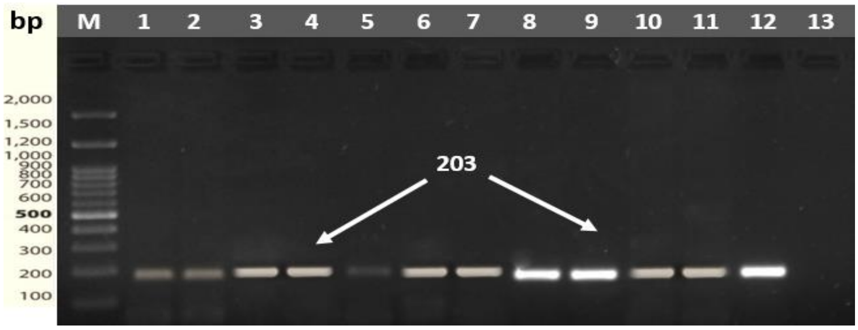

Fig. 5: PCR amplification products for *clf* B gene at product size of 203 bp for MRSA isolates

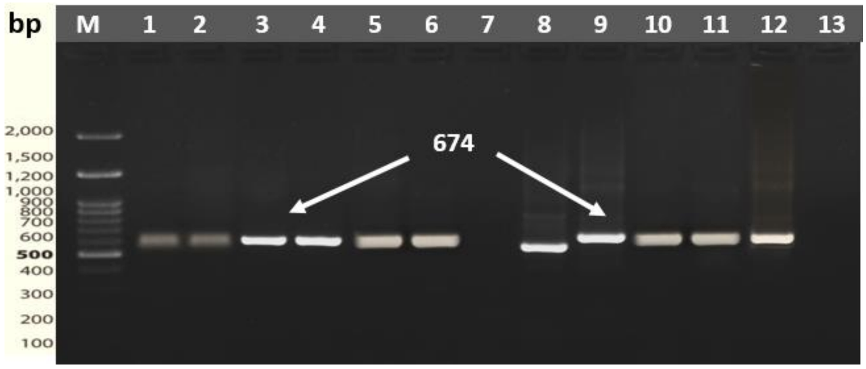

Fig. 6: PCR amplification products for *coa* gene at product size of 674 bp for MRSA isolate

As shown in **table 4,** for MRSA isolates, the frequency of presence of *clf* A, *clf* B, and *coa* genes in the same sample was 65.85%, while the frequency of the genes *clf* A and *clf* B was 80.49% and the frequency of the *clf* B, and *coa* genes was 65.85%.

Table 4: Frequency rates of *clf* A, *clf* B, and *coa* genes in MRSA isolates

N.	Gene	Frequency out of 41 isolates	
		No. of Isolates	%
1	*clf*A + *clf*B + *coa*	27	65.85
2	*clf*A + *clf*B	33	80.49
3	*clf*B + *coa*	27	65.85

DISCUSSION

Staphylococcus aureus is considered an invasive species and an important factor in the development of diseases in animals and humans because of the various virulence factors it contains (**Sakr *et al.*, 2018; Kwiecinski and Horswill, 2020; Hu *et al*., 2021**). For those isolated from animals and humans, studying the distribution and spread of *S. aureus* will contribute to developing surveillance and expanding the epidemiological concept of *S. aureus* infections worldwide. Here, animals play a major role as reservoirs for the emergence of new species that cause human diseases with the potential of spreading, and this is what is indicated by **Scherrer *et al., (2004);* Crespo-Piazuelo and Lawlor (2021) and El-Ashker *et al.*, (2022**).

To estimate the potential risks of *S. aureus* strains, especially zoonosis MRSA, it is necessary to know the differences between the types of *staphylococci* in terms of antibiotic resistance, their possession of virulence factors, and their ability to cause infection. This is what was indicated by **Stapleton and Taylor (2002) and Chen and Wu (2020**). In this study, the highest percentage of *S. aureus* isolation based on the *nuc* gene was recorded in cattle, followed by humans and buffaloes (54%, 40%, and 32%, respectively), while the cattle isolation rate reached 24% for MRSA. A lower isolation rate of *S. aureus* was reported in Algeria by **Mairi *et al.*, (2019)** and **Agabou *et al.*, (2017),** ranging from 18% to 35% and 5.4% to 7.6% for MRSA.

The possession of the *mec*A gene by *S. aureus* adds to the bacteria another new mechanism for resistance to antibiotics, especially methicillin. It is an inevitable criterion for diagnosing MRSA molecularly, as mentioned by **Lee *et al.*, (2004**). The highest percentage of MRSA isolation based on the *mec*A gene was recorded in buffalo (30%), followed by humans (28%), then cattle (24%). This increases the belief in the high rate of nasal transmission in the presence of animals as a reservoir, which may be the reason for the bacterial shedding into the farm environment and their transmission to workers and breeders and vice versa, as the rates of bacterial isolation of MRSA from cattle, amounting to 24%, were higher than the results of the study conducted in Iran by **Rahimi *et al.*,** (**2015**), Tunisia by **Gharsa *et al.*,** (**2015**), Algeria by **Agabou *et al.*, (2017**), and Norway by **Mørk *et al.*,** (**2012**) with rates of 5.06%, 1.3%, 15%, and 13.9%, respectively. On the other hand, it was less than what was recorded in Dohuk Governorate by **Abdulrahman and Abdulrahman** (**2023**), Kingdom of Saudi Arabia by **Alzohairy** (**2011**), Greece by **Papadopoulos *et al.*, (2018)**, France by **Gharsa *et al.*, (2012)**, Nigeria by **Igbinosa *et al.*, (2016),** and India by **Kumar *et al.*, (2017)** with the rates 62%, 50%, 54%, 44%, 38%, and 31.43%, respectively.

To our knowledge, there is only one research study on the spread of nasal MRSA in buffalo that was conducted by **Kumar *et al.*, (2017)**, in which he indicated an isolation rate of 46.9%, which is higher than the rates we obtained in our study (30%). It was previously believed that MRSA was transmitted primarily from humans to animals through the hands and front nostrils, but it was soon proven that transmission occurs in both directions. When animals are exposed to the bacteria and colonize, they can become a reservoir for the organism and can be transmitted to other animals, their keepers, and their handlers, as the researchers indicated by **Weese (2005); Stone (2012**); **Richardson (2019)**. The possible transmission of the pathogen and its spread to humans occurs through contact with animals through breeding, treatment, domestication, nasal secretions, movement, and mixing in society. Additionally, it can spread through the contamination of meat by workers and handlers who are infected.

In a study conducted on hospitals patients in Germany by **Cuny *et al.*, (2015)**, on infections associated with the MRSA, they confirmed an increase in infections from 14% in 2008 to reach 23% in 2011, which was explained by the transmission of the bacteria to and from farms to surrounding communities, in Nineveh Governorate, the number of animal breeding fields is increasing around urban centers, where small numbers of animals are present, and most of them are in places where the working classes are located, where the two-way transmission of germs occurs directly through contact, or through the polluted environment; water sources, soil, air, and exposure to manure and contaminated tools; in addition to the animal's places located in an environment that lacks infrastructure for shelter and sanitation or even by consuming contaminated animal products as recorded by **Kadariya *et al.*, (2014**).

The use of antibiotics also contributes to the exchange of antibiotic resistance and virulence genes of *S. aureus* between humans and animals, which may lead to the emergence of new strains, as mentioned by **Hiltunen *et al.*, (2017**) and **Wang *et al.*, (2019).** Among the virulence factors that were highlighted in the current study, which are considered to be microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), are clumping factors, which include Clumping Factor A (*Clf*A), which was found in 100% of the isolates. *Clf*A is a protein fixed to the cell wall of *S. aureus* and is considered a virulence factor for many species as it facilitates the colonization of protein-coated biomaterials and enhances the adhesion to the blood plasma protein fibrinogen (Fg), as reported by **O´Brien *et al.*, (2002) and Zecconi and Scali (2013**).

Another clumping factor expressed by *S. aureus* is Clumping Factor B (*clf*B), which was detected in 33 out of 41 isolates (80.49%). It is a surface protein that causes skin abscess by binding to the structural protein of the host's skin; Loricrin, which constitutes more than 70% of the keratinized envelope and acts as a barrier protective for the stratum corneum, as reported by **Peacock *et al.*, (2002) and Koreen *et al.*, (2005**).

Another clumping factor expressed by *S. aureus* is Clumping Factor B (*Clf B*), which was detected in 33 out of 41 isolates (80.49%); is a surface protein that causes skin abscess by binding to the structural protein of the host's skin; and Loricrin, which constitutes more than 70% of the keratinized envelope and acts as a barrier protective for the stratum corneum, as reported by **Peacock *et al.*, (2002) and Koreen *et al.*, (2005**).

The other factor is the clotting factor *coa*, which was detected in 27 out of 41 isolates (64.28%), which is considered one of the multiple strategies and mechanisms developed by *S. aureus* to intervene locally in the coagulation cascade as recorded by **Hamza *et al.*, (2015**). This study also revealed that the frequency of the virulence genes *clf* A, *clf* B, and *coa*/ the same sample was 65.85%, while for *the clf* A and *clf* B genes, the same sample was 80.49%, while the frequency of the *clf* B and *coa* genes was 65.85%. That is consistent with what **O´Brien *et al.*, (2002)** and **Koreen *et al.*, (2005)** have reported. This study confirms the increasing difficulties in treating diseases caused by MRSA in animals and humans. Therefore, it is supposed to increase preventive measures on farms.

CONCLUSION

The current study concluded that cows and buffalo are considered carriers and potential transmitters of methicillin-resistant *S. aureus*, which makes them risk factors for infection in humans, especially those who are in direct contact with animals. This should be considered an occupational hazard for those working in veterinary fields such as education, health, animal care and production, especially those working in large animal husbandry. Therefore, they must be well educated about the risks of MRSA transmission from and among livestock. This leads us to the need to conduct further studies to determine the possible mode of transmission of pathogenic *S. aureus* between livestock, the environment, and humans. Together, these findings also highlight the need to prevent the transmission of zoonotic pathogens to humans via occupational exposure or consumption of contaminated animal products.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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How to cite this article:
H.Y. Rahma and Dh.M. Jwher, 2024. Detection of *clf*A, *clf*B and *coa* genes in Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolated from Nasal Cavity of Cows, Buffalo and their Breeders in Nineveh Governorate, Iaq. Journal of Applied Veterinary Sciences, 9 (3): 01-10.
DOI:https://dx.doi.org/10.21608/javs.2024.276537.1324