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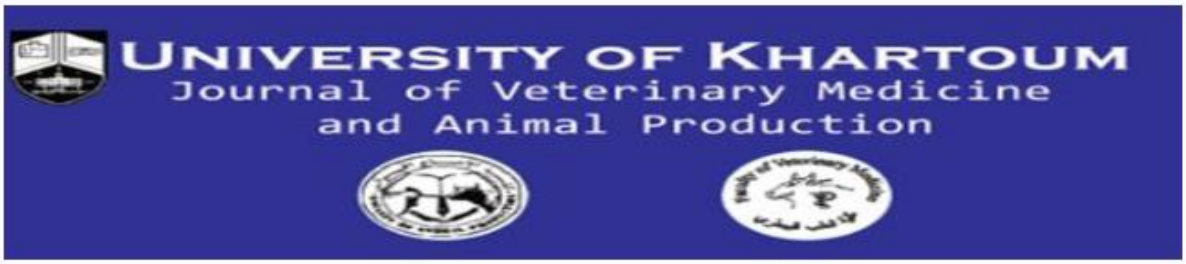

Isolation and Genetic Characterization of Methicillin–Resistant *Staphylococcus aureus* (MRSA) from Animal Products in Khartoum State, Sudan

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Abstract

The aim of this study was to investigate the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) in animal products to contribute in establishing epidemiological data on antimicrobial resistance in the Sudan. Samples (N = 616) of animal products (milk, cheese, chicken and retail meat) were collected from different sources in Khartoum State and subjected to bacteriological and molecular biology techniques for isolation and characterization of *S. aureus*. *S. aureus* was isolated from 62 (10.1%) of the samples with frequencies of 32.7%, 23.3%, 21.5% 7.2% and 4.75% from cheese, chicken meat, retail meat, camel milk and cattle milk, respectively. Methicillin resistance was detected phenotypically among all (100%) cattle milk, 78.6% of retail meat, 71.4% of chicken meat, 50% of camel milk and 23.5% of cheese *S.*

aureus isolates. The methicillin resistance gene (*mecA*) was detected in 3 isolates with one being positive for *mecA* analogue (*mecC*) as well. *mecA* gene detected in these isolates belonged to none of *S. aureus SCCmec* (staphylococcal cassette chromosome mec) known types. Panton-Valentine Leuckocidin (*PVL*), haemolysin (*hlg*) and toxic shock syndrome toxin (*tsst*) genes were detected as 12.9%, 9.7% and 0.0%, respectively, in all *S. aureus* isolates. High prevalence of methicillin resistance among *S. aureus* isolates from animal products, especially from cattle milk indicates widespread of antimicrobial resistance and necessitates adoption of good animal production hygiene practices. The genes responsible for methicillin resistance among phenotypically MRSA but *mecA*/*mecC*-negative strains are yet to be identified.

Keywords: *S. aureus, MRSA, mecA, mecC, PVL, hlg, milk, meat, chicken, camel, cattle*.

المستخلص

هدفت هذه الدراسة إلى استقصاء وجود المكورات العنقودية المقاومة لعقار الميثيسيللين (مرسا) في المنتجات الحيوانية للمساهمة في إنشاء بيانات وبائية حول المقاومة للمضادات الميكروبية في السودان. جمعت عينات (عددها 616) من المنتجات الحيوانية (لبن، و جبن، و لحم فراخ و لحم تجزئة) من مصادر مختلفة في ولاية الخرطوم، و أخضعت لتقنيات علوم البكتيريا و الأحياء الجزيئية لعزل و توصيف المكورات العنقودية الذهبية. عزلت المكورات العنقودية الذهبية من 62 (10,1٪) من العينات بنسب تكرار 32,7٪، و 23,3٪، و 21,5٪، و 7,2٪، و 4,75٪ من الجبن، و لحم الفراخ، و لحم التجزئة، و لبن الأبقار و لبن الإبل، على التوالي. رصدت المقاومة الظاهرية للميثيسيللين في كل (100٪) عزلات المكورات العنقودية الذهبية من لبن الأبقار، و في 78,6٪ من لحم التجزئة، و في 71,4٪ من لحم الفراخ، و في 50٪ من لبن الإبل و في 23,5٪ من الجبن. رصدت مورثة المقاومة لعقار الميثيسيللين (*mecA*) في 3 عزلات بينها واحدة موجبة لنظير *mecA* (*mecC*) أيضاً. لم تنتم مورثة *mecA* لأي من الأنواع المعروفة لعليبة صبغي المكورات العنقودية حاملة *mec* (*SCCmec*). رصدت مورثات كل من بانتون - فالنتين ليوكوسيدين، و حالة الدم، و ذيفان متلازمة الصدمة السمية بتكرار 12,9٪، و 9,7٪، و 0٪، على التوالي، في كل عزلات المكورات العنقودية الذهبية. إن الشيوع الكبير للمقاومة لعقار الميثيسيللين بين عزلات المكورات العنقودية من المنتجات الحيوانية - خاصة من لبن الأبقار - يشير إلى انتشار واسع للمقاومة للمضادات الميكروبية، مما يستوجب تبنى ممارسات صحية جيدة للانتاج الحيواني. تبقى المورثات المسؤولة عن المقاومة لعقار الميثيسيللين في العترات التي هي مرسا ظاهرياً و لكنها سلبية للمورثة *mecA* أو *mecC* في انتظار التعرف عليها.

Introduction

Staphylococcus aureus causes many disease conditions in animals and humans and has the versatility to acquire resistance to multiple antimicrobial agents (Coombs *et al*., 2006). Many isolates of *S. aureus* are frequently resistant to the penicillinase resistant penicillins and are referred to as methicillin- resistant *S. aureus* (MRSA) (Lee, 2003). MRSA was discovered in 1960 affecting two patients and a nurse in a UK hospital (Jevons, 1961). Thereafter, increasing numbers of reports on MRSA were made worldwide identifying this organism as a major cause of nosocomial infections. MRSA causing these infections is referred to as hospital or health-care acquired MRSA (HA-MRSA), after which community acquired and livestock associated MRSA (CA-MRSA and LA-MRSA, respectively) were identified and reported.

Since the emergence of LA-MRSA in 2000s, *S. aureus* in animal products have been widely investigated and raised additional food safety concerns worldwide (Price *et al*., 2012). Although reports on MRSA do not equate to consider this organism a food-borne pathogen, as LA-MRSA and CA-MRSA and even HA-MRSA can be present in/on food intended for human consumption (Wendlandt *et al*., 2013), its presence in food represents a risk to producers and consumers of getting infection through handling and/or processing and especially when food is consumed uncooked.

In the Sudan, a few reports on MRSA in animal products were made and no information is available about its types. Coping with the global and national concern on antimicrobial resistance, the present study aimed at investigating the presence of MRSA in animal products in Khartoum State in the way of establishing molecular and epidemiological data on this pathogen in the Sudan.

Materials and Methods

Samples

A total of 616 animal products samples animal were collected as follows cattle milk samples (N = 400) samples were collected in sterile containers from each quarter of 100 apparently healthy cows in traditional dairy farms in Omdurman, Khartoum North and East Nile localities of Khartoum State; camel milk samples (N = 69) were obtained from animals at al Muwailih Animal Market, Omdurman, to which camels are brought to this market from different areas of the western part of the Sudan. All quarters of the she- camel were milked in one sterile container. Cheese samples (N = 52, 46 white and 6 braided) were purchased from small groceries. Retail meat (minced meat, mortadella and sausages) samples (N = 65) were purchased from supermarkets: either frozen (produced by meat processing factories) or fresh (made locally in the supermarket). Chicken meat samples (N = 30) were obtained by swabbing the chicken carcasses before being cut in poultry meat processing laboratories. The groceries/ supermarkets, farms and animal market were assigned randomly for sample collection. Care was taken during sampling to avoid contamination from the environment or from the investigator herself.

Isolation of *S. aureus*

The samples were inoculated on mannitol salt agar plates before being incubated aerobically overnight at 37 °C as follows: from the milk samples, 100 µl was streaked on the agar plate; 2 g of meat and cheese samples were suspended in 8 ml of normal saline from which 100 µl was used for inoculation; the swabs of chicken meat were streaked over the agar plates. From the growth culture, after Gram's staining, suspected *S. aureus* colonies were sub-cultured on nutrient agar and tested for catalase and coagulase. Confirmation of identification of the isolates as *S. aureus* was made by PCR.

Susceptibility of *S. aureus* isolates to methicillin was determined by testing their growth on Mueller-Hinton agar (HiMedia, Mumbai, India) plates using disc diffusion method with 5 µg methicillin discs (Bioanalyse Ltd., Ankara, Turkey). After 24 hrs incubation at 37 °C, the results were interpret according to standard protocols (CLSI, 2017).

Polymerase chain reaction (PCR)

The DNA was extracted from pure bacterial cultures by QIAgen mini blood kit (Qiagen, Hilden, Germany) or by boiling, after incubation of the colonies with 10% lysostaphin (Sigma-Aldrich, Taufkirschen, Germany). Primers for amplification of the *nuc* gene of *S. aureus* (Brakstad *et al*., 1992) were used to confirm identity of the isolates. Primers for amplification of *mecA* (Strommenger *et al*., 2003) and *mecC* (Garcia-Alvarez *et al*., 2011) genes were used for the detection of methicillin resistance in *nuc*- positive isolates. Primers for *PVL* (Lina *et al*., 1999), *hlg* and *tsst* (da Cunha Mde *et al*., 2007) were used to test the isolates for harbouring Panton-Valentine Leukocidin, haemolysin and toxic shock syndrome toxin genes, respectively. PCR amplification conditions described by respective authors were used. *mecA* positive isolates were subjected to multiplex PCR to identify the *SCCmec* type according to Boye and others (Boye *et al*., 2007).

Statistical analysis

Descriptive statistics were used to analyse the results by SPSS release 16.0.0, 2007 (SPSS Inc.).

Results

From the total number 616 samples of animal products, *S. aureus* was isolated from 62 of them representing 10.1% (Table 1). Frequency of isolation of *S. aureus* was 32.7% from cheese, 23.3% from chicken meat, 21.5% from retail meat, 7.2% from camel milk and 4.75% from cattle milk.

Table 1. Frequency of isolation of *Staphylococcus aureus* from animal products in Khartoum State, Sudan

Source	Number of Samples	*S. aureus* (%)
Retail meat	65	14 (21.5)
Cheese	52	17 (32.7)
Poultry meat	30	7 (23.3)
Camel milk	69	5 (7.2)
Cattle milk	400	19 (4.75)
Total	616	62 (10.1)

Phenotypic resistance to methicillin was detected in 65.6% of the tested isolates (one camel isolate was not tested). This resistance was 100% in the isolates obtained from cattle milk, 78.6% in minced and processed meat isolates and 23.5% in white cheese isolates (Table 2). *mecA* gene was detected in only three of the retail meat *S. aureus* isolates, one of them was *mecC*- positive as well.

Table 2: Resistance to methicillin and virulence genes among *Staphylococcus aureus* isolated from animal products in Khartoum State, Sudan

Source of Isolates	No. of Isolates	Resistance to Methicillin: N (%)			Virulence genes: N (%)		
		Disc Diffusion	*mecA*	*mecC*	*PVL*	*hlg*	*Tsst*
Retail meat	14	11 (78.6)	3 (21.4)	1 (7.1)	4 (28.6)	4 (28.6)	0
Cheese	17	4 (23.5)	0	0	0	0	0
Chicken meat	7	5 (71.4)	0	0	0	0	0
Cattle milk	19	19 (100)	0	0	3 (15.8)	2 (10.5)	0
Camel milk	5	2 (50) *	0	0	0	0	0
Total	62	41(66.1%)	3 (4.8)	1 (1.6)	7 (11.3%)	6 (9.7)	0

* Only 4 were tested. *mecA*: methicillin resistance gene, *mecC*: *mecA* analogue. *PVL*: Panton-Valentine leukocidin , *hlg*: haemolysin, tsst: toxic shock syndrome toxin

While *tsst* was not detected in any *S. aureus* isolate, *PVL* gene was detected in 12.9% (4 retail meat and 3 cattle milk isolates) and *hlg* gene was detected in 9.7% (4 retail meat and 2 cattle milk isolates), as shown in Table 2. The 3 *mecA*- positive isolates were positive for both *PVL* and *hlg* genes. The 3 *mecA*-positive isolates were found to harbour non-typeable *SCCmec* element by the multiplex PCR used.

Discussion

Animal products represent the main source of protein in human diet in the Sudan. With increasing population of Khartoum State, demand for more animal products increases,

and accordingly, food safety concerns are to be considered. Although many unpublished studies in the Sudan investigated bacterial contamination of animal products, *S. aureus* has been poorly investigated and limited information on MRSA is available in this concern. The present study aimed at investigating the prevalence of methicillin-resistant *S. aureus* (MRSA) in animal products in Khartoum State to establish epidemiological data on this organism and to contribute to the national efforts in establishing data on antimicrobial resistance.
Results of the present investigation showed that overall prevalence of *S. aureus* in the investigated animal products in Khartoum State was 10%. This percentage may increase to about 19%, if the number of sampled cattle (N=100) was considered rather than the number of samples from udder quarters (400). The frequency of isolation of *S. aureus* from white salty cheese was the highest (32.7%) compared to other products. Salting is the most common and reliable traditional methods used in preservation of Sudanese white cheese (Altahir *et al*., 2014). However, *S. aureus* is tolerant to salt and grows in NaCl concentration up to 15% (Schleifer and Bell, 2009), which might justify its survival in Sudanese salted white cheese. The source of *S. aureus* in this cheese is unknown; it could be due to contamination of milk during milking, handling or packaging by workers, or from the animal itself. In a previous investigations on white Sudanese cheese, *S. aureus* was isolated by Lemya *et al*. (2006). Frequency of isolation of *S. aureus* from white and red meat was more or less the same (23.2% and 21.5%, respectively). Contamination of meat and meat products with bacteria is expected to occur by improper handling after slaughtering and during processing or subsequent storage at elevated temperatures (Todd *et al*., 2008). On the other hand, the frequency of isolation of *S. aureus* from milk samples was the lowest; but, it should be expected. Milk samples were obtained from apparently healthy cows and she-camels and no bacterial growth was detected in 23% and 8.7% of the cattle and camel milk samples, respectively (data not shown). However, in a previous study by Shuiep and others, *S. aureus* was isolated from 7.8% of raw milk samples collected from apparently healthy camels in three different locations in the Sudan (Shuiep *et al*., 2009). This frequency of isolation is more or less the same obtained in the present study (7.2%).
As the aim of the present study was to investigate for MRSA, resistance to methicillin was tested in the isolated *S. aureus* by both phenotypic and genotypic methods. Phenotypic test results showed high prevalence rate (65.6%) of methicillin resistance among *S. aureus* isolates from animal products. The findings that all *S. aureus* cattle milk isolates were resistant to methicillin can be partially attributed to the extensive use of antibiotics in dairy production in organized farms, especially for the treatment of mastitis. But the lowest prevalence (23.5%) of methicillin resistance in *S. aureus* Sudanese white cheese isolates requires interpretation, as cheese is produced from cattle milk. Most of the white cheese in the Sudan is produced in traditional small processing plants in rural areas of the White Nile State, where the dairy production system is different from that in Khartoum State. The production systems in this States are transhumant and semi- sedentary systems, in which the herdsmen do not use veterinary drugs intensively; therefore, unlike the case of traditional dairy farms in Khartoum State, normal microflora and pathogenic bacteria in these cattle are not exposed to antibiotic pressure to develop antimicrobial resistance.
Despite the high prevalence of methicillin resistance among *S. aureus* isolates in this

study, the methicillin resistance gene (*mecA*) was detected in only 7.5% of them. In a concurrent study by the authors of the present study on clinical *S. aureus* isolates, the same findings were also obtained (data not published yet). Similar findings were also reported previously (Elhassan *et al*., 2015; Shore *et al*., 2011). Therefore, other variants of the *SCCmec* element coding for methicillin resistance in these *S. aureus* isolates is likely to be present but are yet to be identified. It is noteworthy that *mecA*- and *mecC*- positive *S. aureus* isolates were obtained from minced and processed meat and that they were also positive for the virulence genes *PVL* and *hlg*. In addition, 2 of them were obtained from frozen meat produced by the same factory (data not shown). Whether the source of these isolates was the animal or human is unknown. As *S. aureus* is part of the normal flora of human and animal skins and mucous membranes, contamination of animal products with MRSA can occur at any stage of the production or processing chains. Knowing the source of *S. aureus* in animal products is useful in designing prevention and control programmes. Therefore, molecular typing of MRSA can be helpful in tracing the origin of MRSA. However, the *mecA*-positive isolates of this study were found to harbour non-typeable *SCCmec* elements. Comparative studies with human isolates using other molecular typing techniques are required for tracing the sources of these LA-MRSA isolates.

The detection of MRSA and *S. aureus* harbouring *PVL* and *hlg* genes in animal products, especially in processed meat produced by meat processing factories is alarming and raises questions about the safety measures in such factories. Contamination with *S. aureus* and other microorganisms can be eliminated or reduced by good hygiene and production practices in addition to good storage conditions of the animal products.

Conclusion

In conclusion, high prevalence rate of methicillin resistance among *S. aureus* isolates of animal products, especially of cattle milk, indicates widespread of resistance to other antimicrobials. Therefore, the data presented in this study contribute to the efforts towards establishing epidemiological data on antimicrobial resistance in the country and are useful for the public health and veterinary authorities in the country, when good animal hygiene and production practices are to be devised. Further investigations are required to identify the genes coding for methicillin resistance in the genotypically- methicillin sensitive, but phenotypically methicillin-resistant *S. aureus* isolated in this study.

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