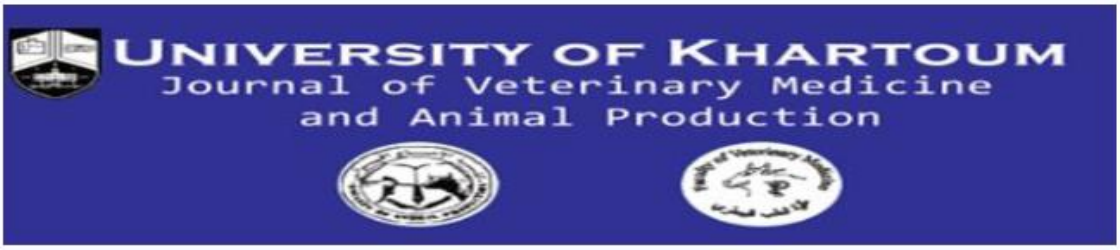
U. of K. J. Vet. Med. Anim. Prod., Vol.9, Issue 2 (2018) page 116 - 122
ISSN: 243456



First Report on *mecC*- positive Methicillin-Resistant *Staphylococcus aureus* Strains in the Sudan

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been reported in many countries as a cause of human infections acquired in hospitals and in the community as well as a cause of animal infections; however, little is known about it in the Sudan, especially in animals and animal products. This study aimed mainly at investigating MRSA in liver abscesses of sheep, as many Sudanese consume raw livers. Intact liver abscesses were collected from sheep slaughtered in 3 abattoirs in Khartoum State and subjected to bacteriological culture for isolation and further molecular characterization of *S. aureus*. Out of 100 liver abscesses, only 4 *S. aureus* isolates were obtained, all of which were resistant to methicillin by the disc diffusion method, but the methicillin resistance gene (*mecA*) was detected in only one of them. When these isolates were tested with primers for the *mecA* gene novel analogue (*mecC*), 3 of them were positive, including the *mecA* positive one. PCR for amplification of haemolysin (*hlg*), Panton-Valentine lecukocidin (*pvl*) and toxic shock syndrome toxin (*tsst*) genes showed that one *mecC*- positive isolate was also positive for *pvl*, but neither *hlg* nor *tsst* was detected in any of these isolates. The results of this study are of public health importance because of the potential zoonotic transmission of *mecC*- positive *S. aureus* strains. Furthermore, this is the first report of *mecC*- positive MRSA strains in the Sudan.

Keywords: *liver abscess, MRSA, mecA*, *mecC*, *hlg*, *pvl*, *tsst*

الخلاصة

سُجِّلتْ المكوراتُ العُنْقُودِيَّةُ المُقَاوِمَةُ لعقارِ الميثسيللين (مرسا) في عددٍ من الأقطار على أنّها مسببٌ للعدوى البشرية المكتسبة في المستشفيات و في المجتمع، و كذلك على أنها مسببٌ لإصابات في الحيوان، إلا أن القليل معروفٌ عنها في السودان، خاصةً في الحيوان و منتجاته. هدفت هذه الدراسة إلى استقصاء مرسا في الدمامل الكبدية في الضأن و ذلك لأن بعض السودانيين يتناولون الكبد نيئاً. جُمِعَتْ دمامل كبديةٌ سليمةٌ من ثلاثةِ مسالخَ في ولاية الخرطوم و أُخْضِعَتْ للتزريع البكتيريِّ لعزلِ المكوراتِ العنقوديةِ الذهبيةِ و مِنْ ثَمَّ توصيفها جزيئياً. من بينِ مائةِ دَمْلٍ كبديٍّ حُصِلَ على 4 عزلاتٍ من المكورات العنقودية الذهبية، كُلُّهُنَّ مُقَاوِمَاتٍ للميثيسيللين بطريقةِ الانتشارِ القرصيِّ، إلا أنَّ مُوَرِّثَةِ مُقَاوَمَةِ الميثيسيللين (*mecA*) وُجِدَتْ في واحدةٍ منهنَّ فقط. عندما اخْتُبِرَتْ هذه العَزْلاتُ بِبادئاتٍ للنظيرِ المُسْتَجِّدِ لِمُوَرِثَةِ *mecA* (*mecC*) كانتْ ثلاثٌ مِنْهُنَّ مُوْجِبَاتٍ بما فيها تلك الموجبة لمورثة *mecA*. أظهر اختبار البلمرة السلسلي لمضاعفة كل من مورثات حالَّة الدم (*hlg*)، و بانتون - فالنتين حالَّةُ الكُرِيَاتِ البِيْضِ (*pvl*)، و زِيفَانِ مُتَلازمةِ الصّدْمَةِ السمية (*tsst*) أنَّ تلك العزلة الموجبة لمورثة *mecC* موجبةٌ أيضاً لمورثة pvl، و لكنْ لم تُوجَدْ مُوَرِّثَةُ *hlg* و لا مورثة *tsst* في أيٍّ من هذه العزلات. نتائج هذه الدراسة ذات أهميةٍ للصحة العامة و ذلك بسبب احتمال انتقال عترات المكورات العنقودية الذهبية الموجبة لمورثة mecC إلى الإنسان. علاوة على ذلك، فهذا أولُ تقريرٍ عن مرسا موجبةٍ لمورثةِ *mecC* في السودان.

Introduction

Staphylococcus aureus is one of the most important bacterial pathogens known today. It has been incriminated in a wide range of illnesses in both humans and animals, in addition to food poisoning. However, *S. aureus* strains can acquire or develop resistance to many antibiotics and to produce many of enzymes and toxins as main factors of causing diseases. Methicillin-resistant *S. aureus* (MRSA) strains have acquired resistance to methicillin by the production of an altered penicillin- binding protein (PBP) known as PBP2' (PBP2a), which has reduced binding affinity to the beta-lactam antibiotics (Reynolds and Brown, 1985; Utsui and Yokota, 1985). This PBP2a is encoded by the methicillin resistance gene (*mecA*), which is carried in the staphylococcal cassette chromosome *mec* (*SCCmec*) elements (Katayama *et al*., 2000). However, another MRSA having a novel *mecA* homologue ($mecA_{LGA251}$), called *mecC*, carried on the *SCCmec*, was identified in 2011; it codes for a different version of PBP2a, which is thought to interfere also with the effects of beta-lactam antibiotics on cell walls. MRSA isolates carrying *mecC* have been recovered from humans, ruminants, pets, and other animals such as rats, seals, and guinea pigs (Paterson *et al*., 2012). Besides carrying the genes coding for resistance against beta-lactams (penicillins and cephalosporins), the *SCCmec* elements also contain some other genes coding for multiple resistance against non-beta lactam antibiotics (Turlej *et al*., 2011).

MRSA was firstly identified in humans in the 1960s associated with nosocomial infections (hospital/ health-care acquired MRSA, HA-MRSA) and later with community acquired infections (community- associated MRSA, CA-MRSA) (Chambers and Deleo, 2009). But, after decades, some strains of MRSA were reported in animals (Farzana *et al*., 2004; Lee, 2003) – termed livestock- associated MRSA (LA-MRSA) – and, accordingly, attracted the attention of the researchers about the possibility of their transmission between animals and humans, which has been documented by some studies (Harrison *et al*., 2013; Steinman *et al*., 2015).

Staphylococcus aureus has been known as one of the bacterial causes of liver abscesses in sheep (Prax *et al*., 2013), a pathological condition leading to condemnations of livers during meat inspection in slaughterhouses to prevent transmission of zoonotic pathogens to humans (Gezu and Addis, 2014). However, improper handling of condemned livers and other organs can lead to contamination of carcasses and other healthy organs with

pathogenic organisms. In the Sudan, people traditionally consume raw livers, especially during Eid Al- Adha fest, in which sheep are slaughtered away from any veterinary supervision. The present investigation was designed to investigate for MRSA in liver abscesses of sheep due to the possible hazards to human health.

Materials and Methods

Samples

A total number of 100 intact abscesses were collected from livers during meat inspection of sheep slaughtered in three abattoirs in Khartoum State (Omdurman, Khartoum, and Khartoum North), Sudan and carried to the laboratory for bacteriological investigations. Animals were brought to these abattoirs from different animal breeding areas of the country.

Isolation of bacteria

The intact abscess capsule was sterilized by the red- hot spatula before being incised by a sterile scalpel blade. Using a sterile loop, the pus was inoculated onto blood agar or mannitol salt agar plates and incubated at 37 ^{o}C for 18 – 24 h. Smears were made from all obtained colonies and stained with Gram stain. Colonies of Gram-positive, catalase-positive cocci were sub-cultured onto nutrient agar plates for purification. Further biochemical tests for identification of isolates were selected based on the Flow Chart for Identification of *Staphylococcus* species (El Sanousi *et al*., 2015). Biochemical tests were performed according to Cowan and Steel's manual for the identification of medical bacteria (Barrow and Feltham, 1993). Isolates identified as *Staphylococcus* spp. were subjected to PCR for confirmation of identity and molecular characterization of *S. aureus*.

Antibiotic susceptibility testing

Susceptibility to methicillin was determined by the presence or absence of inhibition zone around filter paper discs containing 5 µg of methicillin placed on Mueller – Hinton agar plates after being streaked with the suspension of *S. aureus* and incubated at 37 ℃ for 24 h.

PCR

The DNA was extracted from pure bacterial cultures using the QIAgen mini blood kit (Qiagen, Hilden, Germany). Briefly, 5 – 7 colonies were suspended in 100 µl of distilled water to which 10 µl of 10% lysostaphin (Sigma-Aldrich, Taufkirschen, Germany) were added and then incubated at 37 ℃ for 30 min. Further follow-up steps were made according to the manufacturer's instructions. The PCR reaction mixture (20 µl) consisted of 3 µl of DNA template, 10 pmol/ µl each primer, and 15 µl of Maxime PCR ready mix (iNTRON, Seoul, Korea). DNA from isolates identified as staphylococci by conventional bacteriology was subjected to PCR amplification of the *tuf* gene as described previously (Martineau *et al*., 2001). Primers for the *nuc* gene (Brakstad *et al*., 1992) were used to identify *S. aureus* among the *tuf* positive isolates. Primers for amplification of *mecA* (Strommenger *et al*., 2003) and *mecC* (García-Álvarez *et al*., 2011) genes were used for the detection of methicillin resistance genes. 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. The primers used in this study are listed in Table 1. The PCR assays were performed with a Genius thermal cycler (Techne, Cambridge, England) to provide the required thermal cycling profiles. PCR products (7 µl each) were subjected to electrophoresis in 1% agarose gel to which RedSafe stain (iNTRON) was added and visualized under UV illumination and photographed afterwards using a gel documentation system (Major Science, Seoul, Korea).

Table 1. Oligonucleotides used in this study

Primer	Sequence 5' – 3'	Gene	Reference
TStaG422	GGCCGTGTTGAACGTGGTCAAATCA	*tuf*	(Martineau *et al*., 2001)
TStag765	TIACCATTTCAGTACCTTCTGGTAA		
nuc F	GCGATTGATGGTGATACGGTT	*nuc*	(Brakstad *et al*., 1992)
nuc R	AGCCAAGCCTTGACGAACTAAAGC		
mecA1	AAAATCGATGGTAAAGGTTGGC	*mecA*	(Strommenger *et al*., 2003)
mecA2	AGTTCTGCAGTACCGGATTTGC		
mecA_LGA25 1FP	TCACCAGGTTCAACYCAAAA	*mecC*	(García-Álvarez *et al*., 2011)
mecA_LGA25 1RP	CCTGAATCWGCTAATAATATTTC		
lukPV-1	ATCATTAGGTAAAATGTCTGGACATGATCCA		
lukPV-2	GCATCAASTGTATTGGATAGCAAAAGC		
hlg-1	GCCAATCCGTTATTAGAAAATGC	Haemolysin (*hlg*)	(da Cunha Mde *et al*., 2007)
hlg-2	CCATAGACGTAGCAACGGAT		
tst1	ATGGCAGCATCAGCTTGATA	Toxic shock syndrome toxin (*tsst*)	
tst2	TTTCCAATAACCACCCGTTT		

Results and Discussion

Out of 100 liver abscesses, 110 Gram-positive cocci isolates were recovered, out of which only 4 isolates were identified as *S. aureus* (positive for coagulase, xylose, and anaerobic fermentation of 1% mannitol). The 4 *S. aureus* isolates were resistant to methicillin by the disc diffusion method and were positive for the *tuf* and *nuc* genes in PCR. The *SCCmec* was detected in 3 isolates by PCR amplification of *mecC* gene; *mecA* was amplified in one of these 3 isolates as well (Table 2). While a positive PCR result for *pvl* was obtained for one of the *mecC*- positive isolates, none of the isolates was positive for *hlg* or *tsst* genes (Table 2).

Table 2. PCR results of *Staphylococcus aureus* isolates obtained from liver abscesses of sheep slaughtered in abattoirs in Khartoum State, Sudan

Isolate	Genes tested in PCR				
	mecA	***mecC***	***pvl***	***hlg***	***tsst***
3-1	-	+	+	-	-
30	-	-	-	-	-
55-1	-	+	-	-	-
55-3	+	+	-	-	-
3-1	-	+	+	-	-

These results showed that *S. aureus* was incriminated in 4% of liver abscess of sheep slaughtered in Khartoum State. Unpublished data of a similar study (Musa, 2007) from the western part of the Sudan obtained more or less the same results. In that study, the author isolated *S. aureus* from 3.1% of liver abscesses while he was investigating the causes of liver condemnations in Nyala abattoir. Because most of the sheep slaughtered in Khartoum State are brought from the sheep breeding areas in the western parts of the country, it was expected to have results in sheep slaughtered in Khartoum State more or less similar to those of Nyala. However, in a similar study conducted in Iran (Ghadrdan-Mashhadi *et al*., 2006), *S. aureus* constituted 18% of the isolated bacteria. The 4 *S. aureus* isolates of the present study were phenotypically resistant to methicillin, i.e. they were MRSA. Resistance to methicillin is mediated by the *mec* operon, which is part of the staphylococcal cassette chromosome mec (*SCCmec*) element (Katayama *et al*., 2000). The presence of this element was proved by PCR in 3 out of the 4 *S. aureus* isolates. However, the *mecA* novel homologue (*mecC*) was dominant in these isolates. This homologue was firstly identified in the bovine MRSA strain LGA251 (Shore *et al*., 2011). Large scale studies on *S. aureus* collections in the UK and Denmark with whole- genome sequencing of methicillin- resistant, but *mecA* negative strains (García-Álvarez *et al*., 2011; Harrison *et al*., 2013), identified this new homologue in both bovine and human isolates augmenting a zoonotic reservoir. Before the identification of this homologue, some isolates harbouring it were considered methicillin- sensitive strains, as *mecA* was not detectable by conventional or real-time PCR (Shore *et al*., 2011). This might partially explain the results obtained by some investigators (Elhassan *et al*., 2015; Olayinka *et al*., 2010), who detected methicillin- resistance in *S. aureus* by the antibiotic susceptibility testing without being able to detect the *mecA* gene. However, in a parallel study by the same authors on *S. aureus* from human and animal sources, neither *mecA* nor *mecC* was detected in most of the phenotypically methicillin- resistant isolates (data not published yet). It is noteworthy that both *mecA* and *mecC* were detected in one isolate in this study. Further investigations on these methicillin- resistant isolates of *S. aureus* by whole genome sequencing will help in understanding the underlying mechanism of resistance to beta-lactams and to other antibiotics. With the zoonotic potential of *mecC*- positive MRSA, detection of *pvl* gene in one of the 3 *mecC* positive isolates represents a public health hazard, as sequelae of *pvl*-positive infections tend to be more severe than *pvl*-negative *S. aureus* (Gillet *et al*., 2002; Zhang *et al*., 2016).

It is noteworthy that while all *S. aureus* isolated in this study from liver abscesses of sheep are MRSA, liver abscesses in humans caused by MRSA are very rare (Carilli *et al*., 1999; Cherian *et al*., 2016). To the best of our knowledge, no literature is available on MRSA in liver abscesses in animals.

In conclusion, this is the first report of *mecC* positive MRSA in the Sudan. However, despite the low prevalence of *S. aureus* in this study, the finding that all *S. aureus* isolates from a pathological condition in food animals are MRSA represents an important finding which requires further investigations and actions on this pathogen.

Acknowledgements

This study was funded by a grant from the Ministry of Higher Education and Scientific Research, Sudan.

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