CHARACTERIZATION OF TETRACYCLINE-RESISTANT *STAPHYLOCOOCUS AUREUS* ISOLATED FROM BOVINE MASTITIS IN KHARTOUM STATE, SUDAN

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المستخلص

إستخدمت في هذه الدراسة أربع وثلاثون (34) عزلة من الباكتريا المكورة العنقودية الذهبية المعزولة من عينات لبن ابقار مصابة بمرض إلتهاب الضرع في السودان باستخدام طريقة تفاعل البوليميريز المتسلسل الكيفي. فرق إختبار البوليميريز المتسلسل العزلات الي 3 مجموعات، كل بنسبة تشابه فاقت ال 90% بالرغم من ان جميع العزلات قد اظهرت علاقة إرتباط بلغت اكثر من 70%. وجد ان سبعة وعشرون تمثل (79.41 %) من تلك العزلات مقاوم للتتراسيكلين والدايكوسكلين. بالرغم من ان جميع العزلات قد اظهرت حساسية للمينوسكلين. اتضح أن البكتيريا المكورة العنقودية الذهبية المعزولة تحتوي علي عدد 1-3 بلازميد في 30 (88.24%) منها. اكدت نتائج إختبار البوليميريز المتسلسل لبلازميد الحمض النووي ووصمة التهجين للعالم ساوزن ان المقاومة ترجع لوجود *tet* K جين. وجد أن إستخدام انزيم الاقتطاع (Hind III) لقطع بلازميدالحمض النووي في موقع *tet* K جين في عزلات العنقودية الذهبية المقاومة للتتراسيكلين قد فرق العزلات التي تحمل *tet* K جين الي 4 مجموعات. في إحداها وجد ان *tet* K هو PSTs14 بينما الثلاثة الاخريات تحمل *tet* K من PT181. كل النواتج قد حللت بالفصل الكهربائي في هلام الاجار (0.8-1.2%) وحددت بعد صبغها ببروميد الايثيديوم او بدونه.

خلصت الدراسة الحالية الي ان *tet* K جين هو الجين السائد والمسئول عن نقل مقاومة التتراسيكلين في عزلات العنقودية الذهبية المعزولة من الابقار المصابة بمرض إلتهاب الضرع. إذن هنالك حوجة لدراسات تفصيلية لتقييم وتقدير خطر ودور الاستخدام الخاطئ للمضادات الحيوية في الممارسات البيطرية.

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Abstract

Thirty four strains of *Staphylococcus aureus,* isolated from mastitic milk samples and the cow fences in Sudan were used for the polymerase chain reaction (PCR) assay (arbitrary primed PCR), during the present study. The PCR differentiated the isolates into 3 groups, of more than 90% similarity within each. However all the isolates showed more than 70% similarity with each other. Twenty seven (79.41%) of those isolates were tetracycline and deoxocycline resistant. However all the isolates showed susceptibility to minocycline. Plasmids (1-3) were detected in 30 (88.24%) of *Staphylococcus aureus* isolates. The PCR products of the plasmid DNA and Southern blot hybridization proved that the resistance was due to *tet* K gene. Endonuclease restriction enzymes (Hind III), when used to cut the plasmid DNA at the restriction site of *tet* K gene in tetracycline resistant *S. aureus* isolates, differentiated the isolates carrying the *tet* K determinants into 4 groups. In one of them the *tet* K gene was found to be pST S14, while the other three groups carried *tet* K gene from pT181. Amplification of all products were analysed by electrophoresis in agrose gels (0.8-1.2%) and detected by staining them with or without ethidium bromide.

The present study concluded that *tet* K gene is the common responsible gene for transferring tetracycline resistance among the *S. aureus* isolated from mastitis –infected cows in Sudan. Further detailed studies are needed to evaluate and estimate the risk and role of antibiotics misuse in veterinary practices.

Key words: mastitis, *S. aureus,* PCR, *tet* K gene, tetracycline resistance, cows, Sudan.

Introduction

Mastitis is the inflammatory reaction of the mammary gland, usually caused by bacterial infection and is economically the most important disease in dairy milk production worldwide (El Zubeir *et al.*, 2006). Moreover *Staphylococcus aureus* is recognized as the most important mastitis pathogen (Cabral *et al.,* 2004 and Hamid *et al.*, 2012). *Staphylococcus aureus* represents a major agent of contagious mastitis and is transmitted from cow to cow during milk removal (Akineden *et al.,* 2001). Unlike other major

mastitis pathogens that are associated with transient infections, *S. aureus* causes infection of the longer duration (Ebling *et al.,* 2001). It was found that *S. aureus* infection represents 10 to 12 % of clinical mastitis cases (Tenhagen *et al.*, 2009). However Hamid *et al.* (2012) found 23.4% of clinical cases of mastitis among the *S. aureus* infected cows in Khartoum State.

The treatment of *S. aureus* mastitis is associated with poor success (Sutra and Poutrel, 1990), leading to a relatively high culling rate (Boerlin, *et al.,* 2003). The lack of appropriate mastitis therapy results in the development of antibiotics resistant bacteria (El Zubeir *et al.*, 2006). Use and often abuse or misuse of antimicrobial agents has encouraged the evolution of bacteria towards resistance, resulting into therapeutic failure (Straut *et al.*, 1995). The most frequent antibiotic resistance profile of strains isolated from animals and foods were penicillin/ tetracycline, penicillin/ lincomycin and penicillin/ lincomycin/ tetracycline (Kaszanyitzky *et al.,* 2003). As a consequence of the extensive use of tetracyclines, the occurrence of tetracycline resistance has been observed in a wide variety of bacteria (Schwarz *et al.*, 1998). Most of bacteria resistant to tetracycline have acquired different tetracycline resistant genes (Roberts, 1994 and Schwarz *et al.*, 1998). Roberts (1994) reported that, up to now, 18 different tetracycline resistant genes (*tet*) have been described, many of which are associated with either self- movable or movable elements. This tetracycline resistance is widespread among the different Staphylococcal species (Schwarz *et al.*, 1998 and El Zubeir *et al.*, 2007). The respective plasmids genes were commonly found to be located on small plasmids between 4.5 and 11.5 kb (Lyon and Skurray, 1987 and Schwarz *et al.*, 1992). These plasmids carried either *tet* (K) or *tet* (L) genes products specified membrane- associated effux systems (Lyon and Skurray, 1987 and Schwarz *et al.*, 1992).

Several techniques, of which DNA fingerprinting has proven to be easy and reliable and can be used to differentiate *S. aureus* (Lipman *et al.*, 1996). Molecular methods such as PCR- based DNA fingerprinting or hybridization have also successfully been used for *S. aureus* identification and typing (Marcos *et al.,* 1999). Hence the aim of the present study was to characterize and to demonstrate the types of resistance genes responsible for

tetracycline resistance among *S. aureus*, isolated from dairy farms in Khartoum State, Sudan.

Material and Methods

Bacterial strains and antibiotics susceptibility test

Thirty four strains of *Staphylococcus aureus*, isolated from mastitic milk samples (29) and air of their fences (5) were chosen for the present study. Twenty seven of the isolates (79.41%) were resistant to tetracycline and dexocycline. However they were mincocycline sensitive. All isolates were identified by their colonial morphology, Gram stain, catalase activity, staphytect plus (Oxoid) and coagulase test (Difco labrotories). The susceptibility of the isolates was examined by the agar diffusion assay. The selection of the antibiotics was done following the work of Schwarz *et al.* (1989) and El Zubeir *et al.* (2006). Zones of growth inhibition around the antibiotic disks were evaluated after overnight incubation on Muller-Hinton agar at 37° C.

DNA preparations and gel electrophersis

Isolation and purification of the chromosmal DNA were done according to the method of Jordens and Hall (1988), while the modification of the alkaline lysis procedure, described by Schwarz *et al.* (1989) were followed for the isolation and purification of plasmid DNA. Both were visualized in agrose gels (0.8%).

Polymerase chain reaction (PCR) for epidemiological typing

The concentrations of chromosmal DNA were estimated and then 5 ngm were used for the PCR assay. Polymerase chain reaction fingerprinting technique (arbitrarity primed PCR) was used to demonstrate DNA polymorphisms among the tested isolates (Gräser *et al.*, 1993). Two single primers were used as single primers in the PCR experiments to amplify DNA fragments in the genome of *S. aureus* (Primers 1 (5´ -d[GTAGACCCGT]-3´), produced by Amersham Pharmcia Biotech; Ready To Go; and Primers 1 (5´ -d[TCACGATGCA]-3´), produced by TIB Molbiol. Germany).

Amplification products were analysed by electrophersis in agrose gels (1.2%) and detected by staining with ethidium bromide. Then the strains were identified by their DNA polymorphism patterns (Gräser *et al.*, 1993). The

photographs (type 667 plaroid film) of PCR gels were scanning (Flalbeded scan, Mustek, Germany). Gel images were evaluated using scan Pack 3.0 soft ware (Biometra, Germany). The obtained PCR profiles were examined for numbers of bands and their sizes.

Restriction endonuclease analysis

Restriction endonuclease enzymes (Hind III, Gibco), were used according to the recommendations of the manufacturers to cut the plasmidal DNA of the isolates. The resulting fragments were separated by gel electrophoreses.

Southern blot hybridization

Plasmids and their restriction fragments which cut by Hind III were transferred to nitrocellulose membranes by capillary Southern blot (Southern, 1975). Detection of *tetK* gene was done using 0.76 kbp Clal-kpnl fragment of pT 181 (Khan and Novick, 1983). This gene probe was labelled by the non radioactive enhanced chemilumescence kit (ECL, Amersham-Bucher, Braunschweig, Germany). The isolates were screened with *tetK/L* PCR assay, using primers of TL 32: 5′-CCTGTTCCCTCTGATAAA-3′ (located in *tetk* from pT 181 and TK1) 5′-CAAACTGGGTGAACACAG-3′ (non-degenerate oligonuclotide of TL2; especially for *tetk*), and the PCR products were verified as reported by Pang *et al.* (1994).

Results

Polymerase chain reaction (PCR) for epidemiological characterization of the isolates

The PCR could differentiate the isolated strains into three groups, each of more than 90% similarity. However all the isolates showed more than 70% relationship and even some of the strains of the different groups were found to have a close relationship with each others Most of the similar isolates have the same size of bands that ranged from 331 to 2090. They differed in possessing some of the bands, which ranged from 2335 to 6904 and 204 to 323bp. The numbers of bands were differed among the isolates (9 to 13 bands), as shown in Fig. 1.

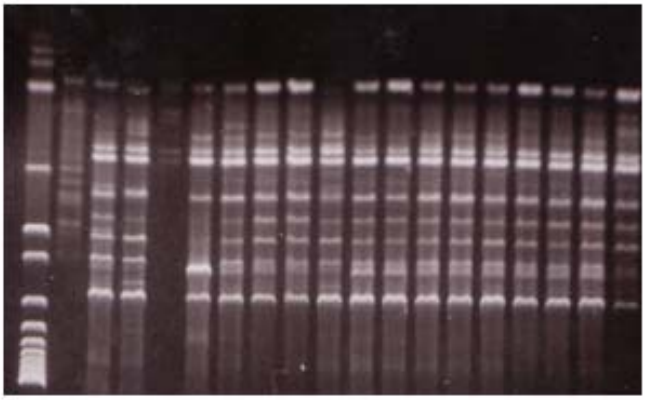

Fig. 1: Arbitrarily primed PCR for *Staphylococcus aureus* isolates from Sudan

Antibiotics susceptibility test

Tetracycline resistance was found in 27 (79.41%) of the tested *S. aureus* isolates. However all the isolates were found to be susceptible to minocycline. Plasmids were detected in 30 (88.24%) of these cultures varying in numbers from 1 to 3 per culture as shown in Table 1. However some of the isolates, which carried the plasmids, were found to be susceptible to Tetracycline (Table 1).

Table 1: Numbers of the plasmids found in the *S. aureus* isolated from Sudan

Numbers of the plasmids	Numbers of *S. aureus* isolates
0	4*
1	7
2	10
3	12
4 or more	1**

* = Tetracycline sensitive
** = *tet k* gene from pSTs14

Polymerase chain reaction (PCR) for *tet* K gene detection

The PCR product of plasmid DNA, using combined probes of *tet* K/L (kPn1 fragment of pT 181), revealed the presence of either *tet* genes (Fig. 2). Southern blot hybridization with *tet* K and *tet* L proved that it was *tet* K.

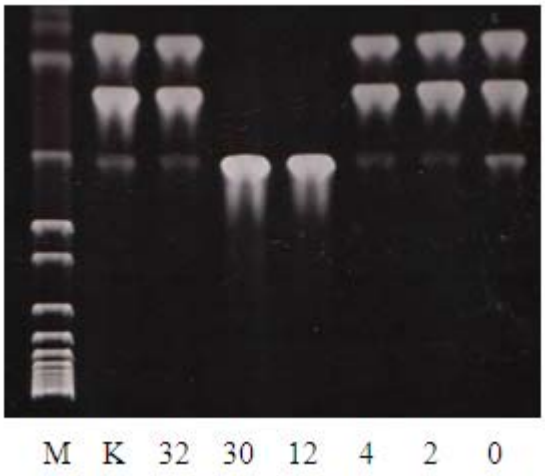

Fig. 2: kPn1 restriction for PCR plasmid fragments *tetK/L*

Endonuclease restriction enzymes

Hind III restriction enzyme was used to cut the plasmid DNA at restriction sites of *tetK* gene (0.65, 1.56 and 2.35) in tetracycline resistant *S. aureus* isolates. All isolates showed the same restriction patterns, with the exception of one isolate, which carried the same plasmid but at different

location. The PCR product of one isolate was never cut with Kpn1. Similarly this finding was obtained from PCR product when hybridized with *tet* K probe (Fig. 2).

The present data could differentiate the isolates carrying *tet K* determinant into 4 groups: one group carrying one small plasmid, including *tet* K gene (1,4,6,7,9,10.13,18,19,21,23,24,26 and 31), the second group carrying in addition to the small plasmid with *tet* K gene, other big plasmids of different sizes (2,11,12,15,16,17,22,27,28,30 and 33). The third group (32) carrying both plasmids, but at different locations from the above mentioned isolates. The fourth group (0), showed both plasmids, however the *tetK* gene was found to be pST S14, while the first three groups carried pT 181.

Discussion

Differentiation of the isolates by their DNA polymorphism pattern (genotype) yielded 3 patterns for coagulase positive *S. aureus*, independent of the primer sets used. Further the strains of *S. aureus* isolated during the present study, revealed a relatedness of more than 70% (Fig. 1), which is in accord with Fitzgerald *et al.* (1997). They concluded that few specialized colonies of *S. aureus* are responsible for the majority of cases of bovine mastitis. Similarly Lam *et al.* (1996) reported that only a limited number of *S. aureus* genotypes were found in each herd. This might be attributable, as they reported, to the contagiousness of the pathogens, causing quarter-to-quarter spread of infections. Moreover, they added another explanation that only a few *S. aureus* strains in the environment have all characteristics that can cause mastitis. The differences in PCR generated polymorphism indicated that some of the strains studied had the same genotype, although they were isolated from different locations and herds around Khartoum State (Table 1). Similarly, Mohamed *et al*. (1993); El Zubeir *et al.* (2006) and Hamid *et al.* (2012) found higher prevalence of *S. aureus* among the different herds in Khartoum State. This is because *S. aureus* is an endemic herd problem in which the infection probably spreads via cow to cow transmission (Lipman *et. al.,* 1996).

The high rate of tetracycline resistance obtained during the present study may be due to the wide spread use of tetracycline among dairy farms in

Sudan (Mohamed *et al*., 1993 and El Zubeir *et al.*, 2006). This was also similar to those of Perreten *et al.* (1998). All tetracycline-resistant isolates were found to carry at least one plasmid (Table 1). However some other isolates were sensitive to tetracycline.

The isolated strains during the present study were found to be sensitive to minocycline, suggesting that it could be either *tet* (K) or *tet* (L) which are both plasmid borne genes (Schwarz and Noble, 1994). They also mentioned that those genes mediated a membrane-associated efflux system, which prevents tetracycline accumulation within bacterial cells.

Hybridization patterns with two separate labelled probes of both *tet* genes revealed the presence of *tet* (K) gene in our isolates. Restriction analysis using Hind III fragments of pT181 as a probe, also confirmed that the isolates carry *tet* (K) genes (Fig. 2). Schwarz *et al.* (1998) reported that the *tet* (K) genes are often located on small plasmids, which can be transduced between members of the same or related *Staphylococcus spp.*.

Conclusion

The present study concluded that most of the isolated *S. aureus* strains, regardless of their isolation sites, location and similarity (relatedness) of their isolation and the numbers of the plasmids DNA that they carried, were tetracycline resistant. This may play a role in the antibiotics resistance hazards, to both animals and humans. Further studies on epidemiological and antibiotics resistance genes are required for *S. aureus* and other food borne disease-causing organisms, in order to minimize public hazards and to state rules and quality control measures for the antibiotics uses and handling for veterinary and human medicines.

References

Akineden, O.; Annemuller, C.; Hassan, A. A.; Lammler, C.; Wolter, W. and Zschock, M. **(2001).** Toxin genes and other characteristics of *Staphylococcus aureus* isolated from milk of cows with mastitis. Clinical Diagnostic Laboratory Immunology, 8 (5): 959- 964.

Boerlin, P.; Kuhnert, P.; Hussy, D. and Schaellibaum, M. **(2003).** Methods for identification of *Staphylococcus aureus* isolates in cases of bovine mastitis. Journal of Clinical Microbiology. 41 (2): 767-771.

Cabral, K. G.; Lämmler, C.; Zschöck, M.; Langoni, H.; de Sa, M.E.P.; Victoria, C. and Da Siliva, A. V. **(2004).** Pheno and genotyping of *Staphylococcus aureus,* isolated from bovine milk samples from Sao Paulo State, Brazil. Can. J. Microbiol., 50: 901- 909.

Ebling, T. L.; Fox, L. K.; Bayles, K. W.; Bohach, G. A.; Byrne, K. M.; Davis, W. C.; Ferens, W. A. and Hillers, J. K. **(2001).** Bovine mammary immune response to an experimental intramammary infection with *Staphylococcus aureus* strain containing a gene for staphylococcal enterotoxins C1. Journal of Dairy Science, 84: 2044- 2050.

El Zubeir, I. E. M.; Kanbar, T.; Alber, J.; Lämmler, C.; Akineden, O.; Weiss, R. and Zschöck, M. **(2007).** Phenotypic and genotypic characteristics of methicillin resistance from *Staphylococcus intermedius* isolated from clinical specimens during routine veterinary microbiological examinations. Veterinary Microbiology, 121: 170-176.

El Zubeir, I. E.M.; Kutzer, P. and El Owni, O.A.O. **(2006).** Frequencies and antibiotic susceptibility patterns of bacteria causing mastitis among cows and their environment in Khartoum State. Research Journal of Microbiology, 1(2): 101- 109.

Fitzgerald, J. R.; Meaney, W. J.; Hartigan, P. J.; Smyth, C. J. and Kapur, V. **(1997).** Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows. Epidemiol. Infect., 119 (2): 261-269.

Gräser, Y.; Klare, I.; Halle, E.; Gantenberg, R.; Buchholz, P.; Jacobi, H. D.; Presber,, W. and Schonian, G. **(1993).** Epidemological study of an *Acinetobacter baumannii* outbreak by using Polymearase chain reaction fingerprinting. J. Clin. Microbiol. 31; 2417-2420.

Hamid, I.M.B.; Shuiep, E.S.; El Zubeir, I. E.M. Saad, A.Z. and El Owni, O.A.O. **(2012).** Influence of *Staphylococcus aureus* mastitis on milk composition of different dairy breeds of cattle in Khartoum State, Sudan. World's Veterinary Journal, 2(2): 13-16.

Jordens, S. Z. and Hall, L. M. C. **(1988).** Characterization of methicillin-resistant *Staphylococcus aureus* isolated by restriction endonuclease digestion of chromosmal DNA. J. Med. Microbiol., 27: 117-123.

Kaszanyitzky, E.J.; Janosi, S.; Egyed, Z.; Agost, G. and Semjen, G. **(2003).** Antibiotic resistance of staphylococci from humans, food and different animal species according to data of the Hungarian resistance monitoring system in 2001. Acta Vet. Hung., 51 (4): 451- 464.

Khan, S. A. and Novick, R. P. **(1983).** Complete nucleotide sequence of pT181, a tetracycline resistance plasmid from *Staphylococcus aureus.* Plasmid, 10: 251-259.

Lam, T. J. G. M.; Lipman, L.A. J.; Schukken, Y. H.; Gaastra, W. and Brand, A. **(1996).** Epidemiological characteristics of bovine clinical mastitis caused by *Escherichia coli* and *Staphylococcus aureus* studied by fingerprinting. Am. J. Vet. Res., 57: 39-42.

Lipman, L.A. J.; De-Nijs, A.; Lam, T. J. G. M.; Rost, J. A.; Van-Dijk, L.; Schukken, Y. H. and Gaastra, W. **(1996).** Genotyping by PCR, of *Staphylococcus aureus* strains, isolated from mammary glands of cows. Vet. Microbiol., 48: 51-55.

Lyon, B. R. and Skurray, R. **(1987).** Antimicroibial resistance of *Staphylococcus aureus*, genetic basis. Microbial Rev. 51: 88 -134.

Marcos, J. Y.; Soriano, A. C.; Salazar, M. S.; Moral, C. H.; Ramos, S. S.; Smeltzer, M. S. and Carrasco, G. N. **(1999).** Rapid identification and typing of *Staphylococcus aureus* by PCR- restriction fragement length polymorphism analysis of the aroA gene. Journal of Clinical Microbiology, 37 (3): 570- 574.

Mohamed, I. E.; Mohamed, G.E. and El Owni, O.A.O. **(1993).** Study on incidence and etiology agents of bovine in Sudan. Proceeding of the second scientific congress, Egypt, Assuit.

Pang, Y.; Bosch, T. and Roberts, M.C. **(1994).** Single polymerase chain reaction for the detection of tetracycline-resistance determinants Tet K and Tet L. Mol. Cell. Probes, 8: 417-422.

Perreten, V.; Giama, N.; Schuler- Schmid, U. and Teuber, M. **(1998).** Antibiotic resistance gene in coagulase-negative Staphylococci isolated from food. Syst. Appl. Microbiol., 21(1) : 113-120.

Roberts, M.C. **(1994).** Epidemiology of tetracycline-resistance determinants. Trends in Microbiology, 2: 353-357.

Schwarz, S.; Roberts, M.C., Werckenthin, C.; Pang, Y. and Lange, C.. **(1998).** Tetracycline resistance in *Staphylococcus spp.* from domestic animals. Vet Microbiol., 63 (2-4): 217-227..

Schwarz, S. and Noble, W. C. **(1994).** Tetracycline resistance genes in staphylococci from the skin of pigs. J. Appl. Bacteriol., 76: 320-326.

Schwarz, S.; Cardoso, M. and Blobel, H. **(1989).** Plasmid-mediated chloramphenicol resistance in *Staphylococcus hyicus*. J. Gen. Microbiol. 135: 3329 - 3336.

Schwarz, S.; Cardoso, M. and Wegener, H. C**. (1992).** Nucleotide sequence and phylogeny of the *tet*(L) tetracycline resistance determinants encoded by the plasmid pSTE1 from *Staphylococcus hyicus.* Antimcrob. Agents Chemother., 36: 580- 588.

Southern, E.M. **(1975).** Detection of specific sequences among DNA fragments separated by gel electrophoresis. Mol Biol., 98(3): 503-517.

Straut, M.; Surdeanu, M.; Oprisan, G.; Otelea, D. and Damian, M. **(1995).** Antibiotics and antibacterial resistance. A few elements of genetic basis for this relationship. Roum. Arch. Microbial. Immunol. 54 (4): 241-254.

Sutra, L. and Poutrel, B**. (1990).** Detection of capsular polysaccharide in milk of cows with intramammary infection caused by *Staphylococcus aureus.* American J. Vet. Res., 51: 1857- 1859.

Tenhagen, B.A.; Hansen, I.; Reinecke, A. and Heuwieser, W. **(2009).** Prevalence of pathogens in milk samples of dairy cows with clinical mastitis and in heifers at first parturition. Journal of Dairy Research, 76: 179- 87.