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Molecular detection of Methicillin Resistance in *Staphylococcus aureus* Isolated from raw cow milk in Ghebaish Locality,West Kordofan

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Abstract:
The occurrence of MRSA organisms is one of the problems that facing the world now in treatment of bacterial infections. The antibiotic resistance is common among pathogenic bacteria associated with both community acquired and nosocomial infections, *Staphylococcus aureus* is notorious for its ability to become resistant to antibiotics. The aim of this study was to detect the specific mecA gene in the *Staphyllococcus aureus* isolates. *Staphylococci* strains were isolated from raw cow milk in Ghebaish Locality and were identified with conventional methods and APi system. A total of 200 samples were collected and 33 *Staphylococci* strains (16%) isolates were obtained. Bacterial DNA was extracted from each isolate using boiling method. PCR was used to detect mecA gene. The results indicate that 23 isolates were positive to mecA gene (69%).The mecA gene sequence of *S. aureus* showed high similarities with those recorded in gene bank. The study concluded that, Bacterial DNA extracted from *Staphylococcus aureus* reflected appearance of mecA gene which was responsible of resistance to methicillin.
Key words: *Staphylococcus aureus,* MRSA, mecA gene, PCR.

Introduction:

Staphylococcus aureus can infect any part of the body; it causes some diseases in humans and animals, ranging from skin infection, food poisoning, brain abscesses and outbreak in post operative wound infection (Kenneth, 2008). In cows, it causes some important diseases including mastitis (clinical and sub clinical) and respiratory tract infection, skin sepsis, tick pyemia in lamb and contagious skin necrosis (Kinight, 1999). *S. aureus* is one of the major causes of serious infections, passively colonizing human skin and nasal passages of healthy individuals; although this opportunistic pathogen colonizes without causing diseases (Kloos *et al*., 1992).One of the specific features of *S. aureus* is its ability to acquire resistance to antibiotics (Ohta *et al*., 2004).

Antibiotics have multi use in animals e.g. for treatment of infectious diseases and also they are used as growth promoters. This over use of antibiotics may result in the emergence of resistant bacteria that can be transmitted to human via the food supply. The relationship between the use of antibiotics drugs for animals and the emergence of antibacterial drugs resistant pathogenic bacteria in human is well reported (AitMhand *et al*., 2002).

During the last decade, many studies have demonstrated the extremely high capacity of PCR for specifically detecting bacteria and genes of interest (Salisbury *et al*., 1996). That ability has revealed PCR as a powerful tool in clinical microbiology studies (Cockerill, 1999). Several authors have already shown the feasibility of the PCR methodology for the

identification of staphylococcal strains and the applicability of PCR to the detection of antibiotic resistance genes (Vannuffel *et al*., 1998; Cockerill,1999;Jonas *et al*., 1999).

The first case of meticillin-resistant *Staphylococcus aureus* (MRSA), was reported in the early 1960, which has become an increasing problem in health care centers and communities (Akpaka *et al.,* 2007). Methicillin resistance, in particular hetero – methicillin resistance, in *Staphylococcus aureus* can be difficultto detect by phenotypic methods.

The term methicillin resistant is historically used to describe resistance to any of this class of antimicrobials. The resistance mechanism for methicillin is related to the presence of mecAgene. The mecA gene is located on a mobile genetic element called Staphylococcal Cassette Chromosome mec (SCCmec). Horizontal, interspecies transfer of this element is speculated to be the cause of dissemination of methicillinresistance (Bloemendaal *et al*., 2010). Like methicillin resistance genes, enterotoxin genes are also carried by transmissible genetic elements and therefore, foods contaminated with such staphylococcal strains could play a key role in transmission of antibiotic-resistant enterotoxigenic *Staphylococci* to humans imposing health risk (Chajecka-Weirzchowska *et al*., 2012).

Most clinical isolates of methicillin-resistant *Staphylococcus aureus* have the mecA gene which encodes production of PBP2a, a modified penicillin binding protein with low affinity for β-lactam antibiotics (de Lencastre *et al*., 1994). However, the emergence of resistance in vitro as a result of mutations during subculture on media with increasing methicillin concentrations has also been documented (Berger, Strassle and Kayser, 1989).

Non-methicillin resistant strains of *Staphylococcus aureus* are called methicillin susceptible *Staphylococcus aureus* (MSSA) (Perez *et al.*, 2001).

The mecA gene is a small part of the 21 to 60 kb staphylococcal chromosome cassette mec. This mobile genetic element may also contain genetic structures such as Tn 554, pUB110, and pT181 that also encode resistance to non-β-lactam antibiotics (Ito and Hiramatsu, 1998).

Detection of meticillin-resistance (MR) in the clinical laboratory settings is related to environmental conditions such as temperature, pH, incubation time or salt concentration of the media. Furthermore, conditional or heterogeneous expression of MR can cause ambiguity in routine susceptibility tests (Mohanasoundaram and Lalitha, 2008). These facts justify the need for a sensitive, rapid, and accurate method. Therefore, detection of mec-A gene by PCR method has been considered as the reference method (Prere *et al.*, 2006).

In order to accelerate the procedure of identification in clinical microbiology laboratories, it is very important to have a simple and rapid method for DNA extraction (Berger Bächi *et al*., 1992 ;Anthony *et al*., 1999).

There are several reports in the literature describing methods of extracting DNA from overnight liquid cultures (Tokue *et al*., 1992; Vannuffel *et al*., 1995; Nunes *et al*., 1999). Our aim is used to develop a PCR for the simultaneous identification of *S. aureus* strains and detection of mecA gene using a method of extracting DNA directly from a single colony. For the identification of *S. aureus*, employing of PCR primers targeted to the specific sequence gene. Indeed, no staphylococcal species would yield a PCR fragment using these primers (Martineauf *et al*., 1998).

Currently, multiple-antibiotic-resistant *S. aureus* strains constitute a major health care problem, since they are the etiologic agent of several nosocomial and community acquired pathological infections. For that reason, accurate and fast detection of resistant isolates constitutes a critical goal of clinical microbiology and therefore, PCR assays have become an essential tool in laboratory programs. Although previous reports have evidenced the utility of PCR for the accurate detection of the mecA gene (Tokue *et al*., 1992;Schmitz *et al*., 1997).

The aim of this study were that to detect the specific mecA gene in the *Staphyllococcus aureus* isolates which is responsible for the Methicillin resistance and do sequencing of this gene.

Materials and Methods:

Study design;

Bacteria: Thirty three *S. aureus* were isolated from 200 samples of raw cow milk in Ghebaish Locality – west Kurdufan, Mannitol salt agar and Baird parker's agar as described by Barrow and Feltham (2003) were used as selective media for primary isolation of the *Staphylococci*. The organisms were identified by conventional methods (Barrow and Feltham, 1993), set of biochemical tests like, oxidative fermentation, manitol fermentation, Vogues proskauer and catalase test were performed. The coagulase test was performed to confirm *S. aureus* strains. Identification was also confirmed by using of Analytical Profile Index (API Staph) as described by Layer (2006).

Resistance to methicillin: The *Staphyllococci* isolates potentially harboring mecA gene were those with a positive phenotypic confirmatory test for mecA gene test according to current National Committee for Clinical Laboratory Standards (NCCLS, 2001) criteria. To test for this positive phenotypic, bacteria were grown aerobically in breakpoint concentration of methicillin (SIGMAALDRICH) according to standard method (CLSI, 2009). Resistance was ascribed if flocculent growth was observed after 16 h of aerobic growth at 37°C.

DNA extraction method: DNA was extracted from each of the 33 resistant *Staphyllococci* isolates according to the methods described by Dewanand *et al*. (2007) and Brakstad *et al*. (2009).The genomic DNA was extracted by boiling method. In brief for the extraction of genomic DNA, a single colony of *S. aureus* was taken in 100 µL of distilled water, mixed well and boil for 10 min. After boiling the tubes were placed immediately on ice for cooling followed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant containing DNA is collected which is further used as template DNA.

Oligonucleotide Primers: The oligonucleotide primers used in this study have been previously described (Geha *et al.*, 1994).The primers were obtained from a commercial source (Inqaba Biotechnical Industries (Pty) Ltd., South Africa). The 3-end region of the *S. aureus* specific gene was amplified using A22 nucleotide forward primer (5'-AAA ATC GAT GGT AAA GGT TGG C -3') and
A22 nucleotide reverse primer, (5'- AGT TCT GCA GTA CCG GAT TTG C -3').

PCR amplification conditions: As 10µl of dNTBs(0.4µl), 6.25µl of Tag polymerase(0.25µl), 37.5µl of MgCL2 (1.5µl),62.5µl buffer (2.5µl), 333,75µl of distilled water (13.35µl), 12.5µl of primers forward(0.5µl), 12.5µl of primers reverse(0.5µl). The total volume was 21µl, then completed up to 25µl by 4µl of sample (template). All these reactions performed into PCR tubes (0.2ml capacity).

The amplification protocol was performed with thermocycler (Mastercycler personal®-Eppendorf® Germany) under conditions of an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, anneling temperature of primers is 55°C for 45 seconds and extension at 72°C for 1 min.

The amplified PCR products were resolved by electrophoresis in 1.8% agarose gel at 100 V for 30 min, stain with ethidium bromide and finally visualized under UV trans-illuminator (UVsolo TS® Imaging System, Biometra®, Germany) (Geha, *et al*, 1994) . The gene was identified by sequencing of plasmid. An approximately~533 bp region of the mecA gene was sequenced with the set of primers by (RIKEN BRC –Egypt).

Results:

Thirty three methicillin resistant *Staphyllococcus aureus* isolates (MRSA) were tested for the presence of mecA gene. PCR results showed that 23 isolates were positive to mecA gene (69%) (Fig .1).

The mecA gene sequence of *S. aureus* was also available in gen bank database, which was more similar to this sequence (99% similarity)as in(Fig.2).

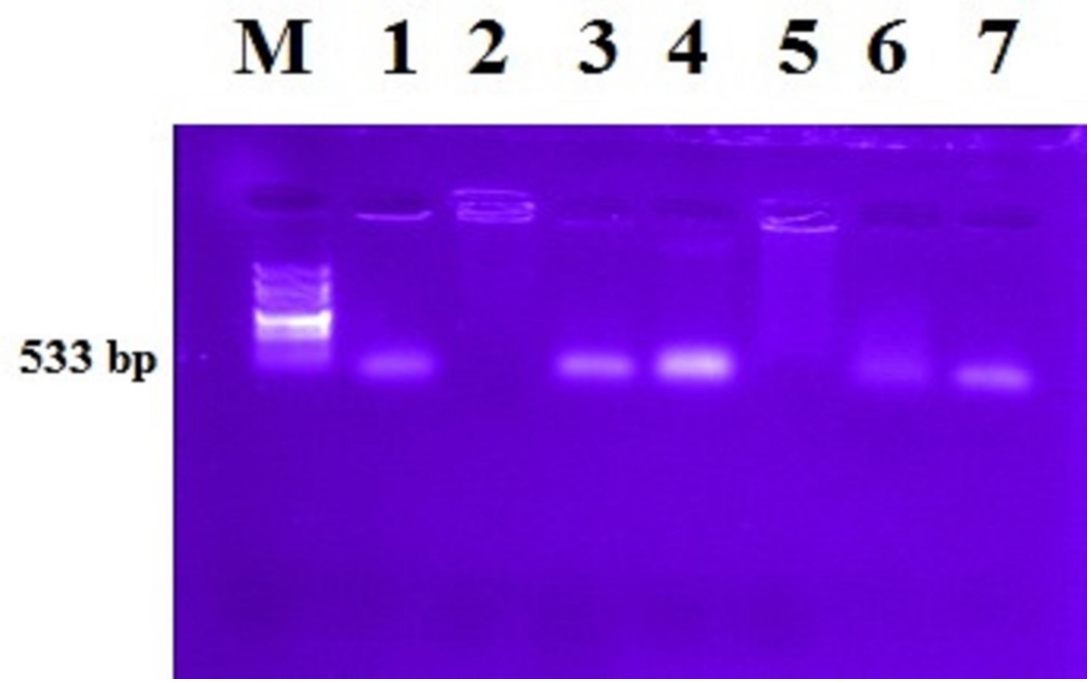

Figure 1. PCR amplification of the mec A gene for Methicillin resistance, Agarose gel (1%) used for separation of PCR products. Amplification of seven DNA extracts of *Staphyllococcus aurus* isolates with mecA specific primers and Marker (M) with **different bands, lanes 1,3,4,6 and 7were positive to the mecA genes lanes 2and 5 were negative.**

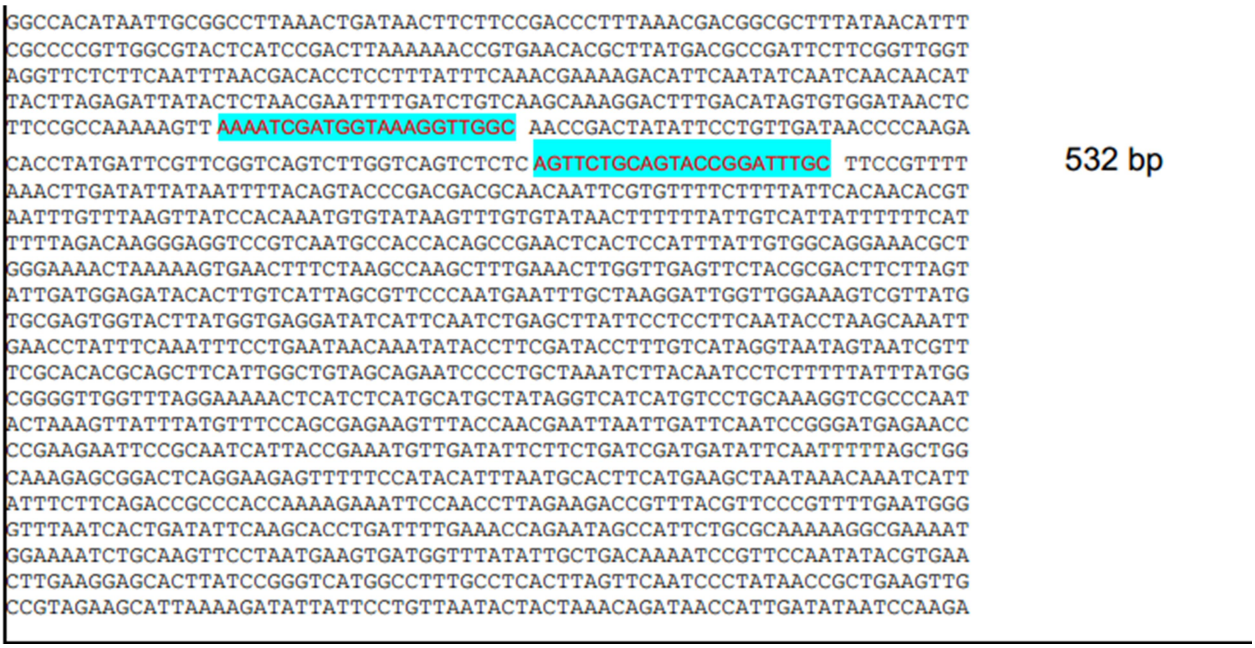

Figure 2. Nitrogen bases sequencing of the *mecA* gene in *Staphylococcus aureus* isolates

Discussion:

Over the past decade, there has been an increase in the rate of infection and diseases caused by *S. aureus* particularly MRSA throughout the world (Sadaka *et al*., 2009). The situation is even more alarming among patients with reduced immunity such as those undergoing chemotherapy or surgery, children, elders and patients with HIV and AIDS as well as animals. *S. aureus* is an important cause of both community and hospital acquired infections resulting in high morbidity and mortality in tropical Africa and understanding the epidemiology of this organism as well as the potential pathogenicity of the infecting strains is important for the design control strategies for the concerned patients. (Awolude *et al*., 2010; Van Rensburg *et al*., 2011).

Molecular techniques remains the most sensitive method in detecting *S.aureus* at both genus and species level and with 100% accuracy in detecting MRSA, when compared with the classical identification method. In addition, for greater detection rates, molecular methods have the shortest turnaround time. Although, molecular testing remains expensive relative to conventional agar based detection (Martineau *et al.,* 2001).

For the strains included in this study, PCR assay was used for detection of methicillin resistance in 33 isolates of *S. aureus*, 23 (69%) mecA positive and 10(30%) mecA negative. This result disagrees with that reported by Tenover *et al.,* (1994) which was 8.7% and less than 93.6% that reported by Al-Khulaif *et al., (*2009) in Saudi Arabia, microbiological susceptibility testing and PCR results did not show concordant results. Detection of the mecA gene was considered the gold standard for MRSA confirmation (Chambers, 1997). The mecA gene, which is responsible for this resistance, is often associated in-vitro with resistance to all ß-lactam antibiotics. MRSA strains are frequently resistant to other classes of antibiotics and results compared with conventional methods of MRSA detection. Previous studies have reported discrepancies, noting that some strains lacking mecA displayed phenotypic resistance to methicillin while others containing mecA showed phenotypic susceptibility (Voss and Milatovic , 1994).

The prevalence of MRSA (69%) identified in this study was higher than that identified in Kwazulu-Natal province and in other major cities in South Africa such as Johannesburg (33%) and Cape Town (43%) (Shittu and Lin, 2006).
In Sudan however, there are paucity of such reports both in *Staphyllococcus* serotypes from human and food of animal origin. This work thus provides an initial database for genes responsible for methicillin resistance in *Staphyllococcus* strains isolated from raw cow milk from Ghebaish locality (west Kurdufan). The findings in this work expose the possible health risk in terms of transfer of drug resistance from these food animal to man. Methicillin as well as other Beta lactams is still the drug of choice in treating some life threatening infections in developing countries (Naas *et al*., 2005). It is important to monitor the emergence of resistant bacteria from animal foods, such animals may be important source of these resistant bacteria which can be spread from their products directly to man, it can jeopardize success of effective treatment thus constituting a potential grave public health hazard.

Conclusions:

Bacterial DNA extracted from *S. aureus* isolates and was screened to the presence of mec-A gene, the study showed high prevalence of mec-A gene activity (69%) among isolates. The results reflected appearance of mec-A gene activity in cows, causing antibiotic resistance of *Staphyllococci* and other species of bacteria.

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