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Detection of Penicillin Binding Protein 2a (PBP2a) in *Staphylococcus aureus* Isolated from Milk Using Serological Assays

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ABSTRACT A total 14 milk samples (7 unpasteurized and 7 pasteurized) were collected and analyzed for characters of *S. aureus*. Based on colonial morphology of the isolates all the samples were positive for the target organism (*S. aureus*). From these samples a total of 16 presumptive isolates from each sample were selected and the resulting 224 isolates were screened for the characters of *S. aureus* by subjecting them to preliminary (Gram staining and catalase test) identification tests. A large proportion 88.8 percent (199/224) were Gram-positive cocci that appeared in clusters and all these isolates were also catalase positive. In addition all these isolates (88.8 percent) were able to breakdown hydrogen peroxide due to the production of the catalase enzyme. When subjected to the MastStaph™ serological assay to confirm their identities a large proportion 59.9 percent (133/224) of the isolates were positively identified as *S. aureus*. All the 133 positively identified *S. aureus* isolates were subjected to another serological assay designed to detect the Penicillin Binding Protein 2a (PBP2a) that codes for the mecA gene in methicillin and oxacillin resistant isolates. Results indicated that about half 49.6 percent (66/133) of these *S. aureus* isolates were positive for the PBP2a protein and eventually the mecA gene and a large proportion 71.2% were isolated from milk obtained from commercial cattle in Molelwane. Despite the fact that all the unpasteurized milk samples were contaminated with *S. aureus* strains, a cause for concern was the fact that this pathogen was also detected in pasteurized milk obtained from some supermarkets in the area. Given that these milk products had not gone past their recommended shelf life, these results therefore indicated that milk products could serve as vehicles for the transmission of *S. aureus* to consumers in the area.

INTRODUCTION

Staphylococcus aureus occurs as normal flora in the gastrointestinal tract of humans and animals (Mahon et al. 1995; Domínguez et al. 2002; Normann and Nass 2005; Akinkunmi et al. 2010). However, some *S. aureus* strains have been found to cause disease in their hosts and this pathogen is currently considered the most common cause of staphylococcal infections worldwide (Matsunaga et al. 1993; Larsen et al. 2000; Olayimka et al. 2005; Becker et al. 2015; Bouchiat et al. 2015; Cuny et al. 2015; Cuny et al. 2016; Sarkar et al. 2016). Diseases caused by *S. aureus* in humans include skin infections, pneumonia, endocarditis, bacteremia and toxic shock syndrome (Tsen et al. 1998; Reacher et al. 2000; Van bambeke et al. 2008). However, in animals the most common disease is mastitis (Waage et al. 1998; Tenhagen et al. 2006; Piepers et al. 2007). *S. aureus* has been detected in undercooked food and dairy products such as milk and cheese; hence these products are known to serve as potential sources for transmission these pathogens to humans (Aramjo et al. 2002; Lee 2003; Normanno et al. 2005; Pesavento et al. 2007; Moraes et al. 2009; Akindolire et al. 2015; Antonios et al. 2015; Bouchiat et al. 2015; Cosandey et al. 2016). Moreover, the presence of this pathogen in food products may result in more severe pathological conditions in individuals who are immuno-compromised, the young and the elderly. *S. aureus* strains have also been found to be a serious problem in communities where proper hygiene is not practiced and among hospitalized patients (Durand et al. 2006). Therefore high incidences of staphylococcal infections have been reported in both developing and developed countries worldwide (Akcam et al. 2006; Becker et al. 2015; Sarkar et al. 2016).

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In South Africa, North West Province and the Mafikeng area in particular, individuals in most rural communities have cattle farms and since these are usually low income earners they obtain milk from the animals. Cow milk is high in nutrients such as vitamins, proteins, lactose, fat, minerals and water and therefore, plays an important role in assisting individuals to meet their nutrient requirements (Michaelidou 2008; Nongonierma and FitzGerald 2015.). However, it has been reported worldwide that foods of animal origin, particularly milk and dairy products, are often associated with food-borne diseases if proper sanitary and health care procedures are not implemented during the production and marketing of these products (Jørgensen et al. 2005; Havelaar et al. 2010; Newell et al. 2010; Akindolire et al. 2015; Antonios et al. 2015; Cosandey et al. 2016). This is mainly due to the fact that milk may serve as an excellent medium for the survival and growth of many different types of pathogenic microorganisms hence it is regarded as a potential vehicle for the transmission of bacteria to humans including staphylococci (Normanno et al. 2007; Huong et al. 2010; Antonios et al. 2015; Cosandey et al. 2016). In these rural communities individuals who milk animals usually do not practice the required farm management techniques and hygiene. The current paper is designed to isolate and identify *S. aureus* from milk obtained from a commercial farm and some shops in the Mafikeng area. A further objective is to determine the presence of *mecA* antibiotic resistant determinants in confirmed *S. aureus* isolates using a serological assay. The aim of the paper is to isolate, identify and determine the antibiotic resistant profiles of *S. aureus* from milk.

MATERIAL AND METHODS

Sample Collection

A total of 7 milk samples were collected from a commercial dairy cattle farm in Molelwane and communal farm in Madibe village. Moreover, 7 pasteurized milk samples were also bought from some supermarkets in the Mafikeng area, North-West Province, South Africa. Approximately 50 ml of milk samples were collected from milking containers and aseptically placed into sterile bottles. In situations where the samples were collected directly from the animals, swabs placed in 70 percent ethanol were used to clean the teats before milking. These samples were properly labeled, kept on ice and transported to the Microbiology Laboratory of the North-West University - Mafikeng Campus for analysis.

Media used for laboratory analysis

Mannitol salt agar (MSA) obtained from Biolab and supplied by Merck, South Africa was used for selective isolation of *S. aureus*.

Laboratory Analysis

On arrival in the laboratory, ten-fold dilutions was prepared for each sample using 2 percent peptone and aliquots of 100µl from each sample was spread-plated onto mannitol salt agar plates. The plates were incubated aerobically at 37^{o}C for 24 hours. Characteristic yellow colonies on MSA were purified by sub-culturing on new MSA plates and the plates were incubated aerobically at 37^{o}C for 24 hours. Pure colonies were retained identification using morphological and biochemical tests specific for *S. aureus*.

Bacterial identification

Presumptive *S. aureus* colonies were identified using the following criteria:

Gram Staining

Isolates were Gram stained using standard techniques (Cruikshank et al. 1975), which differentiates bacterial species into Gram positive and Gram negative based on the chemical and physical properties of their cell walls (Bergy et al. 1994). Colonies that were Gram- positive cocci, arranged in clusters, were retained for further identification.

Catalase Test

The catalase test facilitates the detection of the enzyme catalase in bacteria isolates. The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide to cells. To perform the test, a microscope slide was placed inside a petri dish present in a biological safety cabinet. Using a sterile inoculating loop, a single pure colony of presumptive *S. aureus* isolate was placed onto the microscope slide. A

drop of 3 percent H_2O_2 was placed onto the isolate on the slide. The petri dish was immediately covered to avoid contamination with aerosols. The slide was observed for the formation of bubbles. A positive reaction was recorded when there was immediate effervescence (bubble formation) and vice versa. To ensure quality control the *S. aureus* ATCC® 25923 used in the experiment.

Confirmatory Identification Tests

MastStaph™ Slide Agglutination Test

Confirmatory identification of the isolates as *S. aureus* was achieved using the Oxoid MastSaph™ rapid latex agglutination test kit that is specific for *S. aureus*. In performing the test a single pure colony of the isolate was placed on the black area of the paper cards provided by the manufacturer using a sterile tooth pick. A drop of the MastStaph™ reagent was added to the colony and both were mixed thoroughly. Positive isolates were identified by the presence of agglutination resulting from the production of protein A. *S. aureus* ATCC® 25923 was used as a positive control in the experiment while *Enterococcus faecalis* (ATCC® 6569) was used as a negative control strain. Results were recorded.

Oxoid Penicillin Binding Protein 2a (PBP2a) Agglutination Test

All isolates were tested for their ability to produce PBP2a, a protein encoded by the *mecA* gene in methicillin resistant *S. aureus* isolates. This was achieved using the Oxoid latex agglutination PBP2a assay that employs a serological procedure for the detection of methicillin and oxacillin resistant *S. aureus*. The test was performed as instructed by the manufacturer (Oxoid, UK). In performing the test, a sterile loop was used to transfer a pure colony into a sterile eppendorf tube and 4 drops of Extraction Reagent 1 was added. The contents of the tube were mixed by vortexing and the tube was incubated using a water bath at 95°C for 3 minutes. The tubes were kept at room temperature for 1 minute. One drop of Extraction Reagent 2 was later added into the tube, mixed well and centrifuged at 1500rpm for 15minutes. The supernatant was used for serological testing of the isolates. An aliquot of 50µl from each supernatant was placed on the test card; a drop of the *S. aureus* positive latex reagent was added and both were mixed thoroughly using sterile tooth picks. Results were read within 3 minutes and isolates were classified as PBP2a positive based on the presence of visible clots on the test cards. Moreover, isolates that produced clots were considered to possess the *mecA* gene based on the phenotypic assay. However, the presence of this gene is usually confirmed through PCR amplification using specific oligonucleotide sequences. *S. aureus* (ATCC 43300) and *S. aureus* (ATCC 29213) were used as positive and negative controls for PBP2a assay, respectively

RESULTS

Preliminary Identification Tests

Isolation and Detection of S. aureus in Milk Samples Using Preliminary and Confirmatory Tests

A total 14 milk samples collected and analyzed for characters of *S. aureus*. The makeup of this was 7 unpasteurized and 7 pasteurized samples. All the samples were positive for the target organism (*S. aureus*). From these samples a total of 16 presumptive isolates were subjected to preliminary identification tests. A total of 224 isolates were screened for the characters of *S. aureus* and a large proportion 88.8 percent (199/224) were Gram-positive cocci that appeared in clusters and all these isolates were also catalase positive. All these isolates (88.8%) were able to breakdown hydrogen peroxide due to the production of the catalase enzyme.

All the 224 isolates were screened using a serological assay to confirm their identities as *S. aureus* strains. A large proportion 59.9 percent (133/224) of the isolates was positively identified as members belonging to *S. aureus* based on the Mast-Staph serological assay. Detailed results on the number of isolates that satisfied the different identification criteria are shown in Table 1. The results obtained in the present paper indicated that *S. aureus* was frequently isolated from milk samples that were analysed. Despite the fact that all the unpasteurized milk samples were contaminated with *S. aureus* strains, a cause for concern was the fact that these pathogens were also detected in pasteurized milk obtained from supermarkets. Moreover, these milk

Table 1: Presumptive *S. aureus* identified using Gram staining, Catalase, MastStaph™

Sample source	*Gram staining (+ve Coccus)*	*Catalase test (+ve)*	*MastStaph™ Test*	*PBP2a test*
Molelwane commercial farm (NT= 106)*	106	106	59	47
Pasteurized (NT= 93)*	93	93	74	19
Total No.	199	199	133	66

products had not gone past their recommended shelf life. The results therefore indicated that these products could serve as vehicles for the transmission of these pathogens to consumers in the area.

Penicillin Binding Protein (PBP2a) for the Detection of the mecA Gene in Positively Identified *S. aureus* isolates

A total of 133 *S. aureus* isolates that were positive based on the MastStaph serological assay were subjected to another serological test designed to detect the PBP2a that codes for the *mecA* gene in methicillin and oxacillin resistant isolates. Results obtained are shown in Table 1. As shown in the Table 1 about half 49.6 percent of the population of *S. aureus* tested were positive for the protein and eventually the *mecA* gene. Contrary to the results obtained for the MastStaph assay, a large proportion 71.2 percent of the isolates that possessed the *mecA* gene were isolated from milk obtained in Molelwane (Table 1).

DISCUSSION

The primary objective of the paper was to isolate and identify *S. aureus* in milk obtained from a commercial farm in Molelwane and some supermarkets in the Mafikeng area. *S. aureus* is a facultative anaerobic gram positive bacterium, occurring as normal flora in humans and animals (Mahon et al. 1995; Khan et al. 1998; Normanno and Nass 2005; Wertheim et al. 2005). Despite this some strains have been found to be pathogenic to their hosts (Matsunaga et al. 1993; Larsen et al. 2000) and are able to cause Staphylococcal infections such as skin infections, pneumonia and toxic shock syndrome (Tsen et al. 1998; Reacher et al. 2000; Van bambeke et al. 2008). *S. aureus* is currently a major cause of food-borne diseases in humans worldwide and this usually result of the consumption of contaminated food products (Le Loir et al. 2003; Scallan et al. 2011). Due to the fact that the staphylococcal food poisoning is usually self-limiting and the patients may recover within 24 to 48 hours after the onset of disease, most cases are therefore not reported to healthcare services. Faced with this reality, the actual incidence of staphylococcal food poisoning is known to be much higher than reported (Jørgensen et al. 2005). Many studies have documented the presence of *S. aureus* in undercooked food and dairy products such as milk and cheese; which therefore suggest that these products are capable of transmitting these pathogens to humans if proper hygiene is not practiced (Lee 2003; Normanno and Nass 2005). Consequently, the detection of food sources that are contaminated with *S. aureus* coupled with the effective and urgent identification of these pathogens is important to curb human infections. Although the source of contamination with *S. aureus* is usually very difficult to identify, it is generally known that individuals who handle food are potential risk factors for transmitting these pathogens (Kluytmans et al. 1995; Jones et al. 2002). Therefore the findings of this paper suggest that the implementation of good hygienic conditions during milking coupled with efficient mastitis control strategies may greatly reduce contamination (Fox 1999).

A further objective of the paper was to detect the presence of PBP2a in all the positively identified *S. aureus* isolates using the PBP2a latex agglutination test kit. This was motivated from the fact that resistance of *S. aureus* to different antimicrobial agents have been reported and is on the increase worldwide (Lowy 2003; Ateba et al. 2010). Antimicrobial resistance is currently an important health problem worldwide (Cosgrove 2006). The development of resistance both in human and animal bacterial pathogens has been ascribed to the extensive therapeutic use of antimicrobials or with their use as growth promoters in food animal production (Hsueh et al. 2005). Methicillin-resistant *S. aureus* (MRSA) was first described in 1961, shortly after the in-

troduction of methicillin (Jevsons 1961). *S. aureus* becomes methicillin resistant by acquisition of the *mecA* gene which encodes a modified penicillin binding protein (PBP2a) that has a low affinity for â-lactams **(**Chamber 1997; Lim and Strynadka 2002; Yang et al. 2006; Moroney et al. 2007; Normanno et al. 2007). The modified PBP2a in MRSA isolates is therefore capable of replacing the biosynthetic functions of normal penicillin binding proteins even in the presence of the â-lactam antibiotics, thereby preventing cell lysis. Consequently, *S. aureus* strains that are producing PBP2a are resistant to all â-lactam antibiotics (Lim and Strynadka 2002).

In the present paper almost half (49.6%) of the isolates possessed the *mecA* gene. Similar observations have been reported in other studies (Shahraz et al. 2012). An interesting observation was the detection of a large proportion of *mecA* positive strains in unpasteurized milk when compared to pasteurized samples obtained from supermarkets.

Since the development of methicillin resistance among *S. aureus* strains, vancomycin has been used as the antibiotic of choice to treat infections caused by MRSA strains but the emergence of vancomycin-resistant *S. aureus* has been reported in some studies (Lee 2003; Ateba et al. 2010). *S. aureus* strains that carry these resistant determinants are known have an increased ability to spread within a population especially if they are also enhanced with virulence genes and this does not only provide therapeutic challenges for clinicians but may poses severe complications to human health (Chakraborty et al. 2011) Therefore, the present paper has revealed the presence of methicillin resistant *S. aureus* in isolates obtained from both unpasteurized and pasteurized milk sold in supermarkets. These isolates may therefore facilitate the transfer of the resistant determinants to humans who consume these products. Considering the problems associated with these antibiotic resistant *S. aureus* strains and most especially the difficulties in the managing of staphylococcal infections (Ito et al. 2003), it is suggested that a paper designed to determine the antibiotic resistant profiles of *S. aureus* isolates in food products such as milk may add more value to these baseline findings. Moreover, it is also necessary to perform routine tracking of the pathogen so as to establish effective control measures.

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