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ORIGINAL ARTICLE

Genetic characterization of antibiotic-resistant *Staphylococcus aureus* from milk in the North-West Province, South Africa



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Abstract Food borne diseases are a major public health concern worldwide. *Staphylococcus aureus* is one of the potential food borne pathogens which causes nosocomial and community acquired infections. In the present study, 74 representative strains of *S. aureus* isolated and characterized in previous study from different milk samples were subjected to random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) and enterobacterial repetitive intergenic consensus (ERIC)-PCR to generate fingerprints to determine the genetic relationships of the isolated strains. A total of 20 RAPD patterns were generated and the number of amplified fragments obtained ranged from 0 to 8 with molecular weight ranging from 250 to 2000 bp. A dendrogram based on fingerprinting pattern grouped isolates into twelve major clusters (I–XII). In the case of ERIC-PCR 9 banding patterns were obtained with amplicons ranging from 1 to 8 and band sizes ranging from 250 to 2000 bp. A total of four major clusters (I–IV) were observed in the dendrogram based on ERIC fingerprints. The discrete banding patterns obtained both from ERIC-PCR and RAPD-PCR showed remarkably the genetic diversity of *S. aureus*. The findings of this study indicate that raw, bulk and pasteurized milk in the North-West Province was contaminated with toxigenic and multi-drug resistant *S. aureus* strains. This emphasizes the need to implement appropriate control

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measures to reduce contamination as well as the spread of virulent *S. aureus* strains to reduce the burden of disease in humans.

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1. Introduction

Staphylococcus aureus is a gram positive cocci commonly present on the skin and mucous membranes of animals and humans (Tong et al., 2015). For the past many years, this organism has established itself as a frequent multiple antibiotic resistant (MAR) bacteria particularly to methicillin and vancomycin that may cause nosocomial or community acquired infections (Boucher and Corey, 2008). *S. aureus* is known to cause a number of pathological conditions in humans and animals that range from mild skin infections, bacteremia, systemic diseases, and osteomyelitis to the more complicated toxic shock syndrome and staphylococcal food poisoning (Abdulgader et al., 2015; Tong et al., 2015). The emergence of MAR *S. aureus* poses therapeutic challenges to the health/veterinary professionals and dairy cattle producers (Hiramatsu et al., 2014). Nowadays, antimicrobial resistance has become a major public health problem. The development of resistance in bacterial pathogens of humans and animals has been ascribed to the extensive therapeutic use of antimicrobials or their use as growth promoters in animal feed production (Silbergeld et al., 2008; Economou and Gousia, 2015). Resistance to various antimicrobial agents in bacteria is attributed to various genes which are present on the plasmids, transposons, chromosome or gene cassettes which are incorporated into integrons (Rychlik et al., 2006).

Food is one of the most common modes of transmission of pathogens to humans and *S. aureus* is one of the important food-borne pathogens (Schelin et al., 2011). Milk is a nutritious food which is consumed by human beings and may serve as an excellent growth medium for various microorganisms including *S. aureus*. Raw or processed milk consist of fats, proteins, vitamins, carbohydrates, minerals etc and therefore is extremely susceptible to microbial spoilage (Prescott, 1999; Anderson et al., 2011). *S. aureus* gets a route into raw milk directly through cows which experience clinical or sub-clinical mastitis and indirectly by unhygienic environment, contaminated utensils used for milking and the milking personnel (Ateba et al., 2010; Anderson et al., 2011). Approximately 10^5 CFU/g count of *S. aureus* is sufficient to produce an enterotoxin that can cause human illness. *S. aureus* contamination of milk can be avoided by heat treatment but it is unable to detoxify the preformed toxin (Barker and Gomez-Tome, 2013). In a few studies, *S. aureus* was even reported in pasteurized milk samples (Schmid et al., 2009; Vahedi et al., 2011). Therefore, regular surveillance is required to examine the raw as well as pasteurized milk samples for the presence of *S. aureus* by traditional as well as molecular techniques. Several molecular techniques are available which can be used to characterize *S. aureus* but enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR) and Random amplification of polymorphic DNA (RAPD) PCR are easier, cheaper and more sensitive as compared to other molecular techniques (Adzitey et al., 2013). In ERIC

PCR consensual region of DNA is amplified by a single primer which yields DNA fingerprints whereas in RAPD PCR arbitrary primer amplified random segments from the target nucleic acid which generate a discrete fingerprinting pattern (Tobes and Ramos, 2005; Shi et al., 2010).

Plenty of reports are available on MAR *S. aureus* contamination in milk worldwide (Morandi et al., 2009; Schmid et al., 2009; Haran et al., 2012; Vahedi et al., 2011; Jamali et al., 2015) but scanty reports are available from the South Africa region (Petzer et al., 2009; Ateba et al., 2010). Therefore, knowledge on the occurrence of *S. aureus* remains limited in South Africa especially in the North-West Province and determination of its prevalence in milk may provide an indication of the health risks associated with the consumption of milk or milk products. Keeping this in view, the present study was designed to study the genetic diversity based on genetic fingerprints of *S. aureus* isolated from different milk samples from the North-West Province of South Africa using ERIC-PCR and RAPD-PCR.

2. Materials and methods

2.1. Study design, sample collection and isolation

In a previous study *S. aureus* was isolated by a cross-sectional study design used for the bacteriological analysis of raw, bulk and pasteurized milk in the North-West Province of South Africa. A total of 200 milk samples were randomly collected from 18 sources (supermarkets, shops and farms) in the four districts of the North-west province in South Africa (Table 1). *S. aureus* was isolated on mannitol salt agar (MSA) media and presumptive isolates were identified by biochemical tests (staining, catalase test, slide agglutination test, DNase test) and molecularly characterized [genus specific 16S rRNA gene amplification and species specific thermonuclease (*nuc*) gene amplification].

In the present study a total of 74 representative *S. aureus* isolates were further subjected to ERIC-PCR and RAPD-PCR to determine the genetic diversity based on DNA fingerprinting data.

2.2. Molecular typing

S. aureus isolates were genotypically characterized using the ERIC and RAPD-PCR protocols. The primer sequences that were used in both typing methods are shown in Table 2. The genomic DNA was isolated using ZR Genomic DNA-Tissue MiniPrep Kit (Zymo Research Corp., Irvine, USA) by following the instructions of the manufacturer.

2.2.1. RAPD-PCR analysis

RAPD-PCR analysis was performed on screened *S. aureus* isolates using the primer M13 (Andrighetto et al., 2001). The thermal cycling conditions for amplification involved 2 cycles

Table 1 Areas of North-West Province of South Africa from which milk samples were collected.

District	Sampling area	Number of samples
Dr. Kenneth Kaunda	Coligny	10
	Carletolville	10
	Potchefstroom	10
	Wolmaransstad	10
Bojanala	Zeerust	10
	Swartruggens	10
	Rustenburg	10
	Luhurutshe	10
Dr. Ruth Mompati	Madibogo	10
	Stella	10
	Setlagole	10
	Vryburg	10
	Taung	10
Dr. Modiri Molema	Disaneng	20 [15] ^a
	Mafikeng	15 [10] ^a
	Mabule	10
	Rooigrond	15 [15] ^a
	Tshidilamolomo	10

^a Number of raw milk samples that were collected from the different areas based on availability.

Table 2 Oligonucleotide primer sequences that were used for RAPD and ERIC-PCR typing of *S. aureus* isolates.

Primer name	Primer sequence (5'-3')	Typing procedure
M13 ^a	GAGGGTGGCGTTCT	RAPD-PCR
ERIC-2 ^b	AAGTAAGTACTGGGGTGAGCG	ERIC-PCR

^a Andrighetto et al. (2001).

^b Vazquez-Sanchez et al. (2012).

of 94 °C for 30 s, 35 °C for 1 min, 72 °C for 2 min; 40 cycles of 93 °C for 30 s, 35 °C for 1 min, 72 °C for 2 min and a final elongation step of 72 °C for 5 min.

2.2.2. ERIC-PCR analysis

ERIC-PCR analysis was performed on *S. aureus* isolates using the ERIC-2 primer as described earlier by Vazquez-Sanchez et al. (2012). PCR was performed for amplification at an initial denaturation at 94 °C for 4 min, 35 cycles of 94 °C for 60 s, 25 °C for 60 s, 72 °C for 2 min and a final extension at 72 °C for 5 min.

2.2.3. Agarose gel electrophoresis of the DNA extracted and PCR products

DNA extracted and PCR products were separated by electrophoresis on a 0.8% (w/v) and 2% (w/v) agarose (Seakme®, Rockland, USA) gel respectively, using Tris-acetate-EDTA (1 X TAE) (40 mM Tris, 1 mM EDTA and 20 mM glacial acetic acid; pH 8.0) on a horizontal agarose gel equipment (Bio-comDirect, Bridge of Weir, UK). Electrophoresis was performed at 100 V for 10 min and later 80 V for 60 min. After electrophoresis gel was stained in 0.1 µg/ml of ethidium bromide (Bio-Rad Laboratories, Canada) solution. A 1 kb DNA ladder (Fermentas, Glen Burnie, USA) was included in all fingerprinting gels as a molecular weight standard. ChemiDoc™ MP Imaging system (Bio-Rad, Hercules, USA) was used to visualize and capture the images.

2.3. Statistical analysis

The genetic profiles generated from the RAPD-PCR and ERIC-PCR were captured using GeneTools (version 3.00.22) software (SynGene, UK). The fingerprints were compared and analyzed with the TotalLab Phoretix 1D Pro software (UK). The presence, absence and intensity of band data were obtained, exported to Microsoft Excel (Microsoft Office, 2003) and used to generate a data matrix. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and complete linkage algorithms were used to analyze the percentage similarity and matrix data. Relationships between the various profiles and/or lanes were expressed as dendrograms.

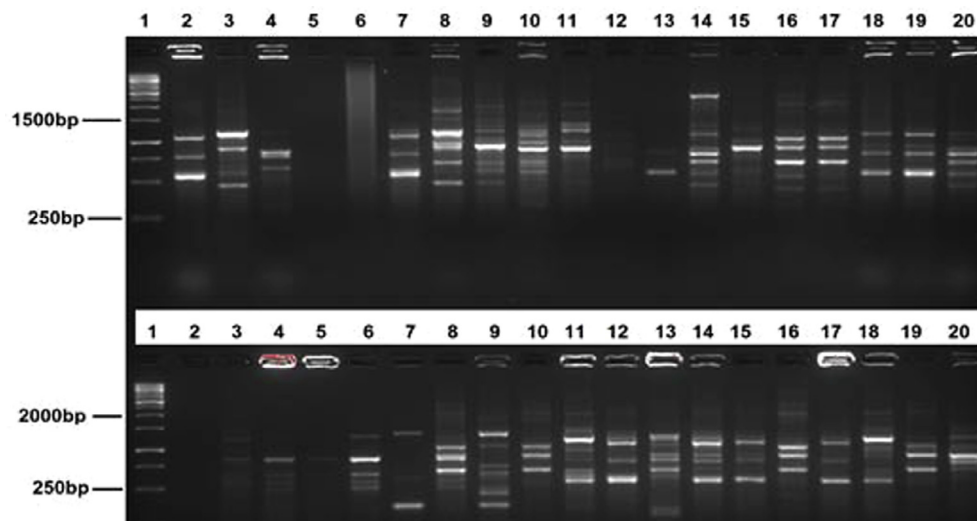


Figure 1 RAPD-PCR profiles of representative *S. aureus* isolates isolated from milk samples from different sampling sites. Lanes 1 = 1 Kb DNA ladder; Lanes 2–20 = A representation of the RAPD profiles for *S. aureus* isolates.

3. Results

The 74 representative *S. aureus* isolates whose identities had been confirmed through amplification of the thermonuclease gene (*nuc*) were subjected to the genotypic typing techniques

(RAPD and ERIC PCR) to determine the similarities between the isolates from different sampling sites. The reproducibility of RAPD and ERIC PCR were calculated for each isolate independently and it was more than 95% for both PCR techniques.

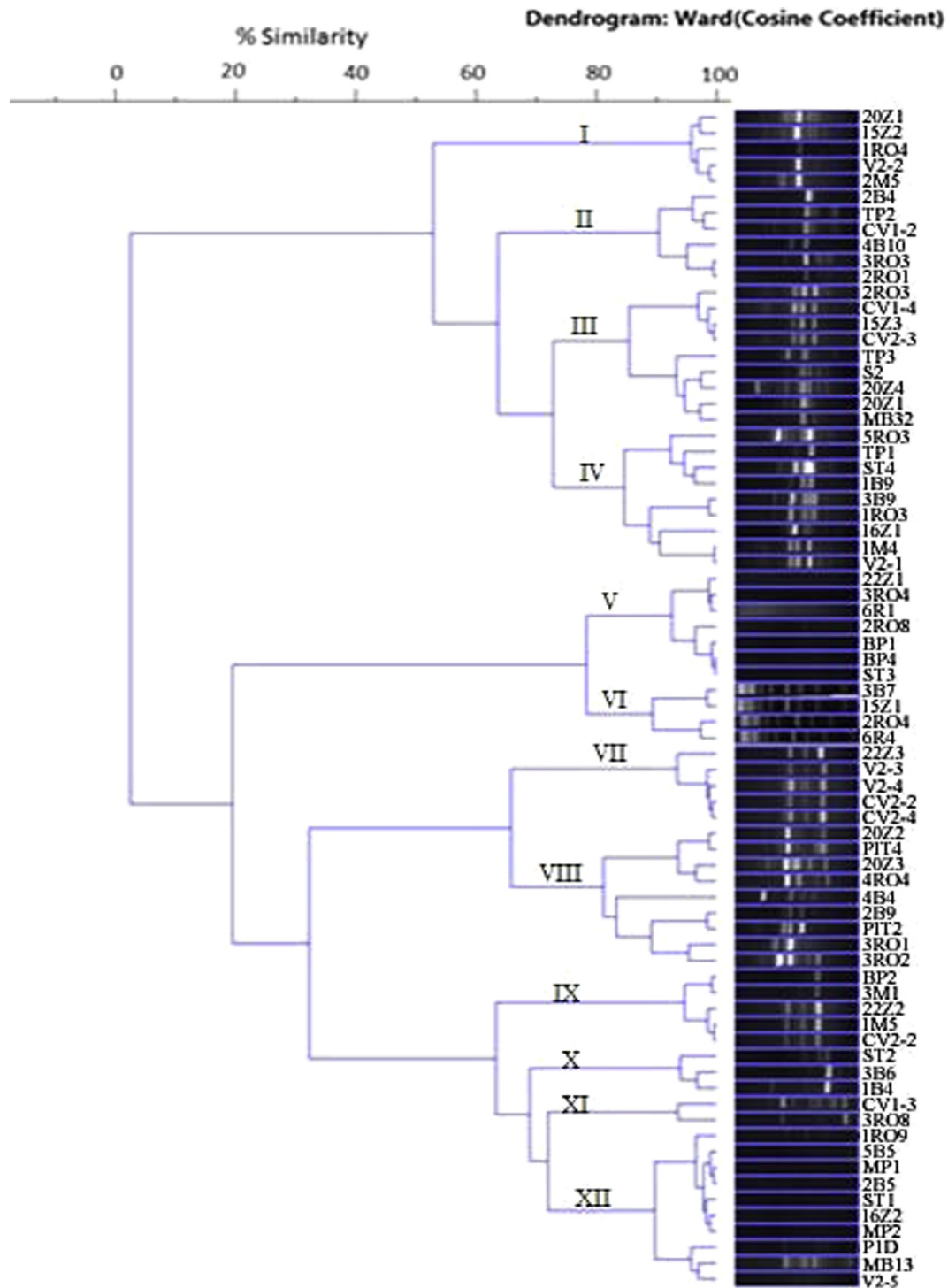


Figure 2 RAPD-based dendrogram showing genetic relatedness among *S. aureus* isolates (Z = Zeerust, RO = Rooigrond, V = Vryburg, MP/MB/M = Mafikeng, B = Disaneng, TP = Taung, CV = Carltolville, ST = Stella, R = Rustenburg, BP = Potchefstroom, PID/PIT = Setlagole).

3.1. RAPD-PCR and ERIC-PCR analysis

The 74 antibiotic resistant *S. aureus* isolates were subjected to RAPD analysis and Fig. 1 shows the DNA fingerprinting profiles obtained. Twenty different band patterns were observed and the number of amplified fragments obtained ranged from 0 to 8 with primer M13. The molecular weight of these fragments ranged from 250 to 2000 bp for different isolates.

Based on these band pattern data, a dendrogram (Fig. 2) was generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). As evident from Fig. 2, twelve RAPD clusters (genotype) with a similarity of 80% were identified. Four profile groups (III, IV, VIII and XII) were the most predominant among the population when compared to the other groups. Profile XII was the most common with 16.2% of the isolates belonging to this group and this was followed by profiles III, IV, and VIII with 12.2% of each isolate. Profile XII had a total of 12 isolates which appeared in all milk types. Profile groups IV and VIII were not present in tank milk but were evident in all other milk types. However, the 9 isolates with profile III were present in all milk types. Isolates from raw milk had the most diverse RAPD-PCR types which appear in 10 out of 12 profile types. Although there was some diversity based on the RAPD analysis among the isolates obtained in the study, a large proportion of the isolates from raw and tank milk showed genetic similarity in clustering patterns (Fig. 2).

All 74 isolates were further subjected to the ERIC genotypic typing technique and the representation of the ERIC-PCR amplicons that was resolved on 2% (w/v) agarose gel is shown in Fig. 3. ERIC profiles produced 9 different banding patterns with amplicons ranging from 0 to 8. The band sizes ranged from 250 to 2000 bp. The dendrogram in Fig. 4 was generated based on the REP-PCR derived fingerprint data using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

At 80% similarity cut-off value, isolates were divided into 4 clusters (designated by I–IV) (Fig. 4). ERIC profile II was the most common with 40.5% of the isolates and this was followed by ERIC-PCR profiles IV, I, and III with 36.5%, 17.6% and 5.4% of the *S. aureus* isolates respectively. ERIC profile group II had a total of 30 *S. aureus* isolated from all milk types. When

the profiles were compared for association of isolates from the different milk sources, profiles I and IV had a similar distribution of strains whereas profile II contained isolates from all milk types except for pasteurized milk. Similar to the RAPD-PCR typing, isolates from raw milk had the most diverse ERIC fingerprinting profiles and this was reflected by the fact that isolates from these milk types appear in all the 4 clusters.

4. Discussion

Food borne diseases are a major public health concern worldwide. *S. aureus* is a commensal and opportunistic pathogen that causes a variety of infections (Lowy, 1998). This organism is potentially versatile having a combination of antibiotic resistance mechanism, invasiveness, and various toxin producing genes. In recent years, it has emerged as a potential food borne pathogen which causes nosocomial and community acquired infections (Kadariya et al., 2014). *S. aureus* flourishes well in various food materials as nutrient rich food serves as an optimum growth medium. Various food materials are implicated in *S. aureus* food disease which may include dairy and dairy products, meat and meat products, poultry products, salad, bakery products etc. (Tamarapu et al., 2001; Argudin et al., 2010).

Milk is one of the important foods which is used by human beings almost daily. As it is rich in nutrients it serves as an excellent growth medium for various microbes (Prescott, 1999; Zakary et al., 2011). Therefore, milk may act as a transmission vehicle for various microorganisms including *S. aureus* from animals to humans. Keeping this in view, a study was conducted previously and *S. aureus* was isolated from different milk samples from the North-West Province of South Africa. A total of 74 representative *S. aureus* isolates were subjected to genetic fingerprinting to determine the genetic relationship of these isolates by using RAPD-PCR and ERIC-PCR. In the present study both these techniques give highly reproducible results. The results of RAPD-PCR showed that isolates of the most dominant cluster 12 (XII) were recovered from all milk types which showed the close genetic relationship of these isolates with each other independent of the type of milk sample. Similar types of findings were also reported

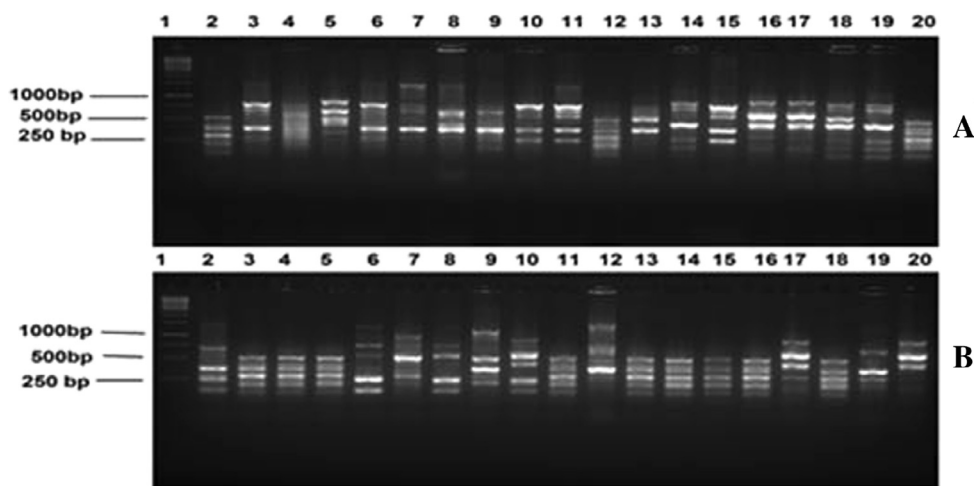


Figure 3 ERIC-PCR profiles of representative *S. aureus* isolates isolated from milk samples from different sampling sites. Lanes 1 and 21 = 1 Kb DNA ladder; Lanes 2–20 A, B = A representation of the ERIC profiles for *S. aureus* isolates.

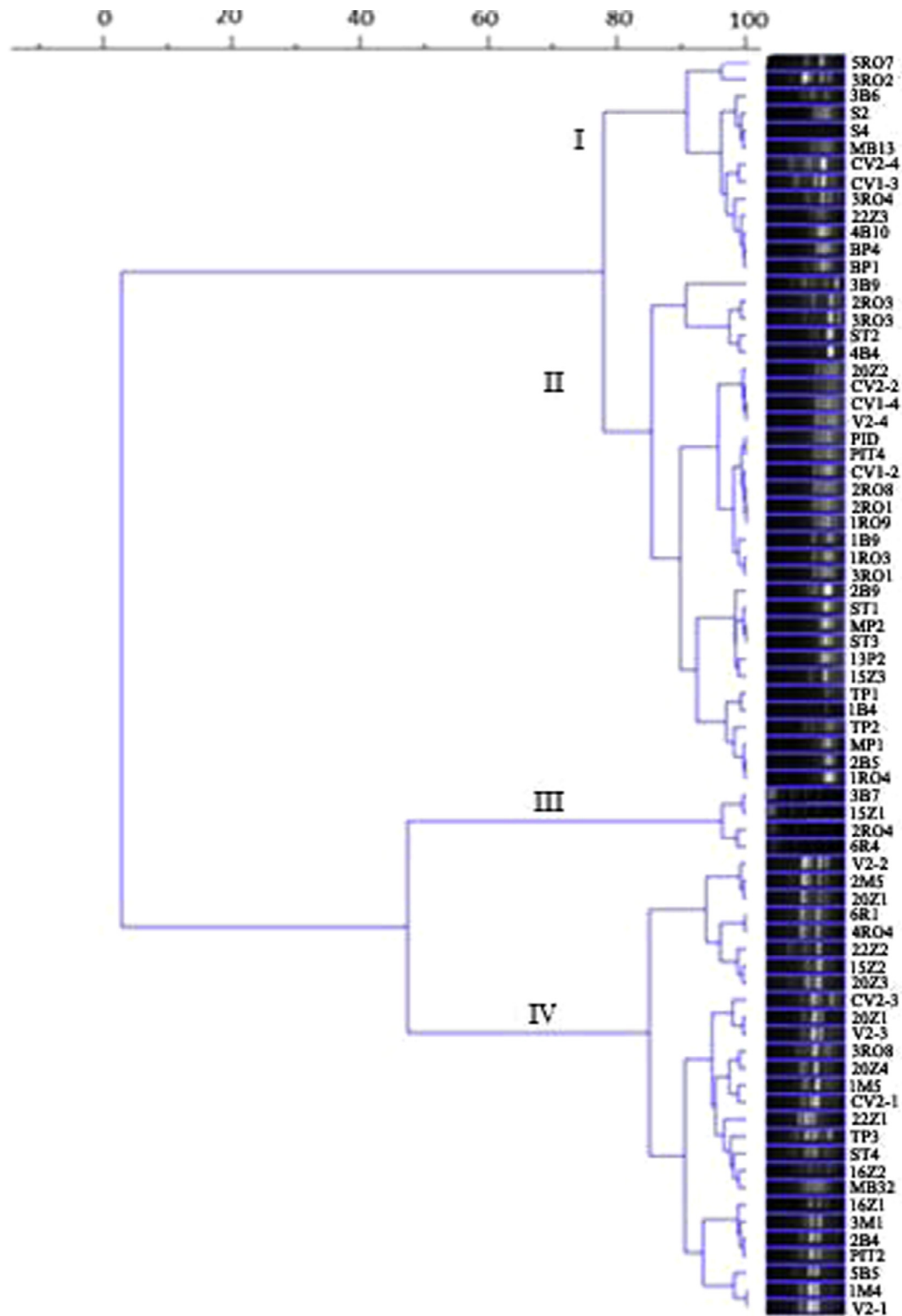


Figure 4 ERIC-based dendrogram showing genetic relatedness among *S. aureus* isolates (Z = Zeerust, RO = Rooigrond, V = Vryburg, MP/MB/M = Mafikeng, B = Disaneng, TP = Taung, CV = Carltolville, ST = Stella, R = Rustenburg, BP = Potchefstroom, PID/PIT = Setlagole).

earlier (Reinoso et al., 2008). Isolates of cluster 4 (IV) and cluster 8 (VIII) were recovered only from raw and pasteurized milk samples. The isolates may be low in number in raw milk which might be the reason that they were not detected in bulk milk

samples. Some isolates were detected in pasteurized milk samples and this could be due to the fact that during heat treatment inadequate heat does not inactivate the isolates which persist after pasteurization and accidentally microbes may be

introduced into milk by handlers hand/unintentionally mixing of raw milk (Seo and Bohach, 2010). The RAPD based dendrogram showed one of the important findings that isolates from raw milk were present in almost all clusters which indicates that raw milk was contaminated with diverse types of *S. aureus* which were genetically distinct and that is why they were distributed in different clusters. The sources of these genetically diverse microbes may be from infected cow milk, udder, handlers hand, utensils used for collection of milk, unhygienic farm conditions etc. (Scherrer et al., 2004; Jorgensen et al., 2005; Morandi et al., 2009; Ateba et al., 2010; Daka et al., 2012; Jamali et al., 2015). Similar findings were observed in the case of ERIC-PCR in which four clusters were observed. Similar to the RAPD, dominant cluster 2 (II) of ERIC-PCR contained isolates from all milk types. Also, cluster 1 (I) and cluster 4 (IV) showed similar distributions of isolates as shown in cluster 4 (IV) and cluster 8 (VII) of RAPD-PCR. Isolates from raw milk samples were distributed in all the four clusters as observed in RAPD-PCR for the same reasons. Earlier workers used these molecular techniques to molecularly characterize bacteria from different food samples (Ruzickova et al., 2008; Morandi et al., 2009; Yingwang et al., 2012; Fendri et al., 2013; Kar et al., 2015). A general observation with these two methods used in the present study was that the clustering of isolates was independent of the type of milk sample and the location. Similar findings were observed earlier where no relationship could be ascertained between fingerprinting pattern and product type/category (Morandi et al., 2009; Kar et al., 2015). The present study showed that RAPD-PCR (12 clusters) has more discriminative power than ERIC-PCR (4 clusters) as DNA bands generated in RAPD-PCR was more as compared to ERIC-PCR. In contrast to discriminative power both the techniques showed the same type of clustering pattern of the isolates. Thus, the combination of both these techniques could be very useful to differentiate *S. aureus* isolates for the purpose of epidemiological surveillance from food samples.

5. Conclusions

Results of the present study depicted the large genotypic diversity of *S. aureus* isolated from different milk samples. Both molecular techniques i.e. RAPD-PCR and ERIC-PCR used in the present study gave highly reproducible results with high discriminative power to differentiate genetic dissimilar *S. aureus* isolated from the same sample. The cause of concern from the present study is that most of the isolates were multiple drug resistant including methicillin and some isolates posed *sec* gene coding for staphylococcal enterotoxin. Therefore, from the public health point of view good hygienic conditions should be maintained in the farm and strict good manufacturing practices (GMP) should be followed in the production area of the milk plant so as to prevent the transmission of *S. aureus* and their associated virulence determinants to consumers.

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