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Isolation, identification, antibiotic susceptibilities and SDS-PAGE protein profiles of *Staphylococcus aureus* strains obtained from She- camel milk eastern Libya

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Abstract

Staphylococcus aureus (*S. aureus*) is instinctively present in milk and dairy products and associated with many outbreaks. Milk is a good substrate for *S. aureus* rise and toxin production. When transmitted to human body this pathogen may affect skin, brain, kidney, liver and several other vital organs. Therefore, the pathological effects of *S. aureus* have been the focus of several recent research works. The objective of this study was to evaluate Antibiotic Susceptibilities of *S. aureus* strains and to Identify their Protein Profiles using SDS-PAGE. *S. aureus* were isolated from different camel milk sample in Libya and identified using microbiological and molecular techniques. The AVIPATH-STAPH test was performed for detection of coagulase and Protein A, both factors are associated with *S. aureus*. All *S. aureus* strains were tested for susceptibility to a panel of 9 antimicrobial disks by the disc–agar diffusion method on Mueller -Hinton agar (oxoid), following the National Committee on Clinical Laboratory Standard. Total Soluble Protein Profiles of *S. aureus* strains were analyzed by SDS-PAGE. Results of this study showed that, all *S. aureus* strains were resistance to the 9 antibiotics tested. Regarding to protein profiles results of *S. aureus* strains, it seems to be different from each other as in Polymorphism Analysis, protein bands and dendrogram analysis. This study confirmed the potential risk of consuming raw she-camel milk, especially upon lack of strict hygienic and preventative measures to avoid the presence of *S. aureus* in milk.

Keywords: Camel milk; SDS PAGE; *S. aureus*; Antibiotic; Susceptibility

1. Introduction

Staphylococcus spp are microorganisms that are commonly present in milk, and they are often associated with food-borne outbreaks due to the ability of some strains to produce thermostable enterotoxins. In addition, they are from the most important pathogen due to the rise in antibiotic resistance, Gabriela *et al.,* (2009). *S. aureus* is a widespread Gram-positive coccus and is considered a prominent cause of human infections globally in health care and community settings. It can be commensal but also a dangerous pathogen, (Wertheim *et al.,* 2005; Musser J and DeLeo, 2015). It can cause skin abscesses, wound infections, pneumonia and septic arthritis, Capparelli *et al.,* (2007). Milk is a good substrate for *S. aureus* and toxin production. The bacterium is also capable of producing several pathological conditions in human [There are numerous animal models of staphylococcal infection have contributed to the knowledge of virulence genes involved in disease. Many researchers reported that *S. aureus* causes osteomyelitis, Sottnik, *et al* (2010); pneumonia Rubinstein, *et al*., (2008), also it causes interaction with the nasal mucosa.

Camel milk is considered an excellent source of nutrients for human and has medicinal properties, Abrhaley A and Leta, (2018). However, it provides a suitable medium for microbial growth and metabolism. Bacteria, in raw milk, can affect

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the quality, safety and consumer acceptance of dairy products. Nonpathogenic bacteria may affect milk quality, Samaržija D*, et al.,* (2012). Thus, different regions have milk quality regulations, including limits on the bacterial count in raw milk, to ensure the quality and safety of the final product. The microbial load in milk, immediately after milking, are concerned by several factors including animal health, apparatus cleanliness and time.

Camel milk is a very rich source for the growth of lot of different species of bacteria that have various effects. Many investigators highlight the beneficial effects of these isolated bacteria including antimicrobial effects probiotic candidates, Soliman *et al.,* (2015); Vimont, *et al.,* (2017). Milk contaminated with harmful bacteria has been linked to several serious diseases including typhoid fever, diphtheria, septic sore throat, scarlet fever, dysentery, Q-fever, and other kinds of food borne illness. Other diseases, including tuberculosis and undulant fever (brucellosis), can be transmitted to people via raw milk from diseased animals. *Staphylococcus aureus* is one of the most common agents in bacterial food poisoning outbreaks.

One of the major problems concern Staph. *aureus* strains is their ability to develop resistance to antibiotics (methicillin, gentamicin and others) also the ability to coagulate citrated plasma and to produce a number of enzymes and toxins which are the main factors of its pathogenicity. However, a limited data is available at present regarding potential of *S. aureus* living in camel milk in Libya and the proposed role of bacterial enterotoxins isolated from raw camel milk. Therefore, the present study aimed to evaluate Antibiotic Susceptibilities of *Staphylococcus aureus* strains obtained from She- camel milk eastern of Libya and to Identification of their Protein Profiles by SDS-PAGE.

2. Material and methods

2.1. Collection of Milk Samples

A total of 220 milk samples from 55 teats of apparently healthy lactating she- camels were collected for investigation from seven different locations in al Jabal al Akhdar, Eastern of Libya (Table 1). The udders of she- camels were thoroughly cleaned with tap water and mopped to dry with clean cloth. The milkers' hands were thoroughly washed with soap and water prior to collection of samples, the first flow of the milk stream was discarded, sample from each quarter were collected separately in sterile test tubes, then tubes were marked. Collection was first done from the near side and then from the offside to avoid contamination of the cleaned teat apices. History and relevant information such as animal number, age, stage of lactation, milk yield and physical condition of the udders were recorded. Samples were kept in an ice box and then transported to bacteriological laboratory, Department of Microbiology, Faculty of Veterinary Medicine, University of Omar – Al Mukhtar Libya.

Table 1 Locations and numbers of teat milk samples

Location	**No of milk samples**		
	No of animals		**No of samples**
	No	**%**	
Gardas	5	9.1%	20
Tanemlo	7	12.7%	28
Om – Alsafsaf	7	12.7%	28
Labrag	5	9.1%	20
Tubrak	14	25.5%	56
Shahat	11	20%	44
Al- Makailie	6	10.9%	24
Total	55	100%	220

2.2. Isolation and identification procedures

Primary isolation procedures were performed in two stages. Firstly, a loop full of each milk sample was inoculated into Nutrient Broth tube. Secondly, test milk samples were directly incubated, considering milk a growth medium. Both inoculated broth tubes and milk samples were incubated at 37 °C for 24-48 hours. Subcultures from the broth tube and

milk bottles were made onto Nutrient agar and Blood agar media, with incubation at 37 °C for 24-48 hours; and pure cultures were obtained by subculturing on the same agar media. Selective and special media such as Mannitol Salt Agar, Baird Parker agar and Deoxyribonucleases agar, were cultured for identification of colonies suspected as genus Staphylococci according to the methods described by (Langlois et al., 1990; Barrow and Feltham, 1993). Identification of isolates was based on colonial characteristics, microscopic features and biochemical reactions. All isolation, identification procedures were done according to (Barrow and Feltham ,1993). Media and reagents used in this study were prepared according to the manufacturer's recommendations.

2.3. AVIPATH-STAPH

The AVIPATH-STAPH test was performed for detection of coagulase and Protein A, both factors are associated with *S. aureus*. When examining the test slides under a strong light source a positive result is indicated by the obvious agglutination pattern of the latex in a clear solution. A negative result is indicated by no change in the latex suspension on the test slide. Agglutination indicates the presence of either coagulase or protein A. Positive AVIPATH STAPH test was confirmed as *S. aureus* by biochemical tests.

2.4. SDS-PAGE Protein Analysis

All steps related to protein work were carried out on ice. Total cellular proteins were extracted from overnight cultures of *Staphylococcus aureus* according to Laemmli (1970) with minor modifications, bacterial pellet of 1.5 ml freshly LB overnight culture was ground in liquid nitrogen and then homogenized in 100 µl of protein extraction buffer (w/v). The mixture was vortexed vigorously, boiled at 95 °C for 5 min. and finally centrifuged for 5 min at 15000 rpm at 25°C. The supernatant was transferred into clean tube and stored in aliquots at (-80 °C) until subsequent analysis by SDS-PAGE analysis. Protein concentration was detected according to (Bradford, 1976), Bradford reagent was freshly prepared right before use. Protein standards were prepared using a range from 5 to 100 micrograms BSA (Bovine Serum Albumin) protein. For protein sample preparation, 19 µl of Bradford reagent was mixed with 100 µl of standard sample or experimental samples and incubated for 5 min, the absorbance was measured spectrophotometrically at 595 nm.

2.5. Total Soluble Protein Analysis by SDS-PAGE

One-dimensional Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) procedure was done according to Laemmli (1970), the sample (100 µl aliquots) was taken from (-80 °C) freezer (thawed only once). The concentration was adjusted to 20 µg in 20 µl in all samples. Loading buffer (10 µl) 2X was added. The prepared sample was boiled at 95 °C for 5 min and cooled at room temperature. The gel casting tray was assembled, then the lower and upper chambers were filled with the upltank buffer. A Hamilton syringe was used to load equal amounts of proteins (20 µl) per well. High range molecular weight protein marker was loaded. Electrophoresis was carried out at 70 volts in Tris/Glycine-SDS-running buffer. After electrophoresis, the gel was stained in 50 ml of the staining solution. For destining, the gel was washed once with ddH_2O and soaked in the destaining solution for 2 hrs with gentle shaking. Destaining solution was changed when turn blue with fresh one until visualizing the protein band(s). The gel was finally placed between two sheets of cellophane membrane and dried on gel drier for 2 hrs and photographed.

2.6. Antibiotic susceptibility testing

Table 2 Antibacterial agents, their abbreviations, potency and source

Antimicrobial agent	Abbreviation	Disc potency	Source
Methicillin	ME	5 mg	Bioanalyse
Gentamicin	GM	10 mg	Difco
Erythromycin	E	15mg	Difco
Cephalothin	KF	30 mg	Difco
Oxytetracycline	OT	30 mg	Bioanalyse
Amoxycillin/ Clavulanicacid	AMC	20 mg	Difco
Penicillin G	P	10 U	Bioanalyse

Five ml of nutrient broth medium was inoculated with each of the test organisms and incubated at 37C^{o} for 18-24h. The cultures were then diluted to densities visually equivalent to that of standard prepared by adding 0.5ml of 1% BaCl2 to

99.5 ml of 1% M percent H2So4 (Bauer et al., 1966). Mueller – Hinton agar plates were dried before inoculation. Using sterile Pasteur pipette the plates were completely covered with the test culture. The excess culture was aspirated, and plates were allowed to dry for 15-30min. Antibiotic discs were applied on the inoculated plates. Plates were then incubated at 37oC overnight. Inhibition zone diameters were measured and recorded in mm. The organisms were recommended sensitive or resistant according to the zone size shown by Hogan et al. (2014). Antibacterial agents, their abbreviations, potency and source are given in (Table2).

3. Results and discussion

3.1. Isolation of *S. aureus*

Based on colonial characteristics, microscopic features and biochemical reactions, all isolates were Gram-positive clusters cocci, positive for both catalase and coagulase tests. The AVIPATH-STAPH test showed obvious agglutination pattern of the latex in a clear solution. Coagulase positive Staphylococci were identified as 2.7% out of the total examined milk samples (220) (Table 3).

Table 3 Identification tests of six bacteria isolates from camels milk

Identification tests	Reaction positive or negative	Inference
Catalase test	+	Active bubbling observed
Heamolysis	+	Narrow zones of clean haemolysis
Coagulase test	+	Firm clot that does not move when the tube is tipped
AVIPATH-STAPH test	+	Obvious clear solution agglutination pattern of the latex in a

Contamination of raw milk with *S. aureus* is mainly due to handling and unhygienic environment; hence the occurrence of these bacteria in milk can cause severe health hazards to people. These findings confirm the finding of Vasavada, (1988), Bonfoh et al (2003) and Soomro et al. (2003). The isolation of the *S. aureus* from camel raw milk in many countries was previously reported by some authors; in Sudan by Shuiep et al. (2009), in Brazil by Fagundes et al. (2010), and in India by Thaker et al. (2013). all the isolates showed β-hemolysis on blood agar media (Figure1), fermentation of mannitol with production of small yellow colonies at the mannitol salt agar (Figure 2), production of black, shiny, convex colonies with entire margins and clear zones on Baird-Parker agar medium (Figure 3), development of Dnase positive colonies (Figure 4).

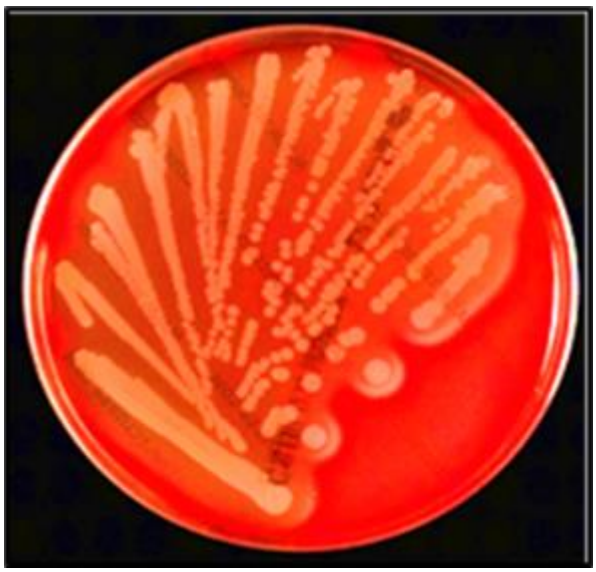

Figure 1 Development of β-hemolysis on 5% sheep blood agar by isolated *S. aureus*

Figure 2 Fermentation of mannitol salt agar by ***S. aureus***

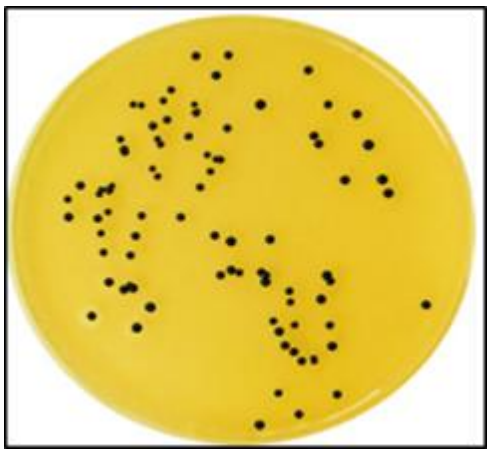

Figure 3 BAGM *S. aureus* produce black, shiny, convex colonies with entire margins and clear zones

Figure 4 *S. aureus* on DNase Agar, appears as a clear zone around the growth

3.2. Antibiotic susceptibility testing

Concerning the susceptibility of isolated *S. aureus* to antibiotics is presented in (Table 4) of the 9 antibiotics tested, ampicillin showed the highest overall resistance followed by Cephalexin, Streptomycin, Neomycin, Cloxacillin, Amoxicillin/Clavulanic acid and Chloramphenicol respectively. Gentamicin and Oxytetracycline were at the same level at 0 level of resistance. Some isolates were intermediate to Cloxacillin (66.7%), Neomycin (33.3%), Chloramphenicol and Cephalexin (16.7%).

Table 4 Antimicrobial susceptibility of *S. aureu* (n = 6) by agar disc diffusion method

Antimicrobial agent	Disc Concentration	Susceptible		Intermediate		Resistant	
		No	%	No	%	No	%
Ampicillin	AM 10 mg	0	0	0	0	6	100
Amoxicillin/Clavulanic acid	AMC 30 mg	4	66.7	0	0	2	33.3
Streptomycin	S 10 mg	2	33.3	0	0	4	66.7
Neomycin	N 30 mg	1	16.7	2	33.3	3	50
Cloxacillin	CX 30 mg	0	0	4	66.7	2	33.3
Gentamycin	G 10 mg	6	100	0	0	0	0
Chloramphenicol	C 10 mg	4	66.6	1	16.7	1	16.7
Oxytetracycline	OX 30 mg	6	100	0	0	0	0
Cephalexin	CL 30 mg	0	0	1	16.7	5	83.3

Results are expressed as a percentage of (n = 6) *S. aureus* isolates susceptible, intermediate/moderately

3.3. Susceptible and resistant, respectively, for each antimicrobial

Knowledge of the pattern of antibiotic resistance among isolates is very important both clinically and epidemiologically. The results of antimicrobial resistance patterns are of great concern due to these predominant bacterial isolates which are highly resistant to commonly available antimicrobial agents. These were stated previously by Ojulong et al. (2009). Sensitivity of the isolated bacteria to gentamycin could explain the possibility of sensitivity to all aminoglycosides. However, in this study isolated *S. aureus* was found resistant to streptomycin as 66.7%, and to neomycin as 50%. These might be attributed to the fact that certain aminoglycosides have a slightly different mechanism of resistance due to their different aminoglycoside modifying enzymes chromosomal mutation and impermeability of membranes (AL

Masaud et al., 1991). The antimicrobial pattern of *S. aureus* was studied by Beatriz et al. (1999), Marais et al. (2009) and Zerfie et al. (2014) who reported susceptibility to ciprofloxacin and ceftriaxone as 95.4% and 80% respectively. They added that *S. aureus* was highly resistant to penicillin G, amoxicillin and nalidixic acid.

3.4. SDS-PAGE Protein Analysis

Protein electrophoresis has been of great value for the delineation of numerous bacterial taxa Vauterin, (1990). Each of the different electrophoretic techniques has its own discrimination level and field of application. It is widely acknowledged that the electrophoretic separation of cellular proteins is a sensitive technique that may provide information on the similarity of strains at and below the species level. Electrophoretic banding patterns of total extracted proteins from different isolates of overnight cultured *Staphylococcus aureus* bacteria done using LB media and grown at 37°C. Fractionation of total isolated proteins was performed using denaturing SDS-PAGE of *S. aureus*. Results of whole-cell protein profiles for six isolate s gave a specific banding Profiles ranging from 5 to 28 bands with low similarity between isolates and within isolates. Comparison of the SDS-PAGE banding patterns showed low degrees of interspecies similarity thus, indicating little correlation among isolates of different staphylococcal species. Figure (5) show different isolate's band. There were very high differences between six isolates (lane 1) *S. aureus* I isolate, (lane 2) *S. aureus* II isolate (lane 3) *S. aureus* III isolate, (lane 4) *S. aureus* IV isolate, (lane 5) *S. aureus* V isolate, (lane 6) *S. aureus* VI isolate).

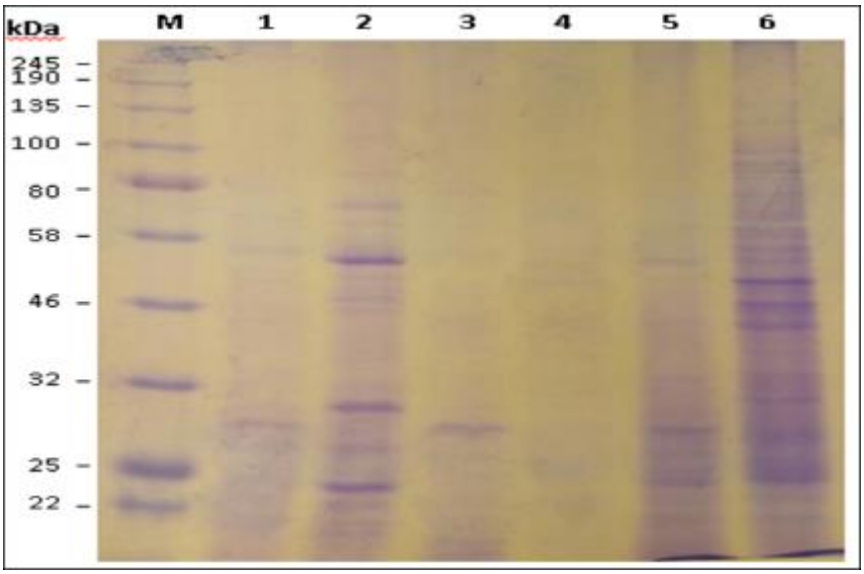

Figure 5 SDS–PAGE of extracellular proteins of six isolates(lane 1 to 6) of *Staphylococcus aureus*

Thomson-Carter and Pennington (1989) analyzed by SDS-PAGE 100 isolates of 9 staphylococcal species and found that interspecies similarity values did not exceed 59.9%, whereas the intraspecies similarity ranged from 76.8% to 100%. Thomson-Carter and Pennington (1989) analyzed by SDS-PAGE 100 isolates of 9 staphylococcal species and found that interspecies similarity values did not exceed 59.9%, whereas the intraspecies similarity ranged from 76.8% to 100%.

3.5. Relationship between flow rate and protein molecular weight

This study obviously showed that the electrophoretic method can provide valuable epidemiological information and may be used in the isolation of *Staphylococcus* strains. Flow rate is the volume of protein which passes per unit time through fractionation of total isolated proteins by denaturing SDS-PAGE. In most contexts a mention of flow rate of each molecular weight of protein. (Table 5) Represent fractionated and analyzed 12 % SDS-PAGE gel of total isolated proteins from *S. aureus* studied isolates by means of calculating the rate of flow (RF) and polypeptide molecular weight in kDa (KD). Previous study by Marais, (2009) of Staphylococcal whole-cell protein profiles and total extracellular protein profiles exhibited the presence of bands 5 and 10 different from other strains and other strains were distinguished by the presence of intense bands 6 and 9.

Table 5 SDS-PAGE banding profiling: relationship between flow rate and protein molecular weight

RF / M	1	2	3	4	5	6
0.14	0.13	0.13	0.45	0.29	0.43	0.13
0.20	0.40	0.24	0.73	0.32	0.45	0.17
0.28	0.45	0.32	0.87	0.38	0.48	0.22
0.51	0.52	0.35	0.90	0.41	0.54	0.24
0.65	0.64	0.38	0.94	0.45	0.64	0.25
0.81	0.73	0.40	0.96	-----	0.73	0.30
-----	0.75	0.43	-----	-----	0.80	0.32
-----	0.83	0.45	-----	-----	0.83	0.35
-----	0.87	0.48	-----	-----	0.87	0.37
-----	0.90	0.50	-----	-----	0.90	0.38
-----	0.96	0.52	-----	-----	0.93	0.40
-----	-----	0.59	-----	-----	0.96	0.43
-----	-----	0.68	-----	-----	-----	0.45
-----	-----	0.75	-----	-----	-----	0.48
-----	-----	0.83	-----	-----	-----	0.50
-----	-----	0.87	-----	-----	-----	0.52
-----	-----	0.90	-----	-----	-----	0.54
-----	-----	0.93	-----	-----	-----	0.56
-----	-----	0.96	-----	-----	-----	0.59
-----	-----	-----	-----	-----	-----	0.64
-----	-----	-----	-----	-----	-----	0.68
-----	-----	-----	-----	-----	-----	0.75
-----	-----	-----	-----	-----	-----	0.80
-----	-----	-----	-----	-----	-----	0.83
-----	-----	-----	-----	-----	-----	0.87

3.6. SDS-PAGE Polymorphism Analysis

There is considerable genetic heterogenecity through natural populations of *S. aureus* (Kapur etal., 1995; Tenover et al 1994). Many different techniques are available for tracing the spread of single *S. aureus* strain of human and animal origin, such as antibiotyping (Pereira et al., 1995) , the biochemotyping (Lang et al., 1999; Lopeset al., 1990) (Lang et al., 1999; Lopeset al., 1990) the phage typing (Witte et al 1988) protein electrophoresis (Costas et al., 1987; Younis A et al., 2000),plasmid profiling (Bauer et al., 1966; Matthews et al., 1992) RFLP-PCR (Goh et al., 1992), RAPDPCR (Fitzgerald et al., 1997; Kapur et al., 1995) and PFGE (Bannerman et al., 1997; et al., 1992) Despite the presence of all these methods, each of these techniques has advantages and disadvantages in their discriminatory power, reproducibility and typeability. The percentage of monomorphic, polymorphic, and unique characterized bands for each *S. aureus* isolate were analyzed in the form of chart showing the genetic material diversity in each and among *S. aureus* isolate(s). Figure (6) confirmed that, the highest percentage of polymorphism characterized bands was in *S. aureus* VI (lane 6) followed by *S. aureus* II(lane 2), *S. aureus* V(lane 5), *S. aureus* I(lane 1), *S. aureus* III (lane 3), and *S. aureus* IV (lane 4), respectively. In addition, there is unique bands characteristic in *S. aureus* VI (Line 6) compared to other strains. Thomson-Carter and Pennington (1989) analyzed by SDS-PAGE 100 isolates of 9 staphylococcal species, and he found low percentage of similarity between *s*trains.

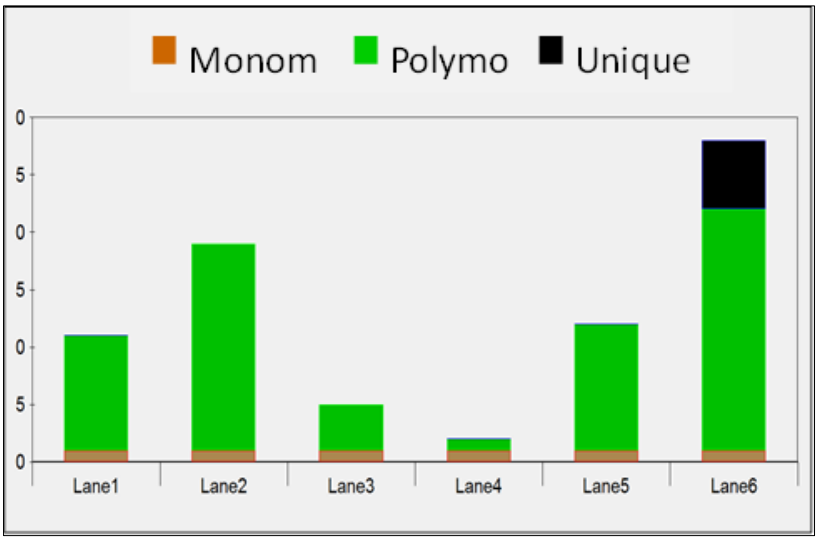

Figure 6 SDS-PAGE Polymorphism Analysis of protein bands of each *S. aureus* isolates

3.7. Cluster analysis as revealed by SDS-PAGE of *S. aureus* isolates.

Different fragments on the gel were numbered sequentially, following that the presence and absence of fragments in each sample was scored (present 1, absent 0) and compared with each other according to the genetic distance method of Nei (Nei et al., 1972). Strains were then clustered by the method of unweighted pair group average linkage (UPGMA). The comparison of the SDS-PAGE banding patterns showed low degrees of interspecies similarity, indicating little correlation among isolates of different staphylococcal species as noted from SDS-PAGE protein profile dendrogram. Results of this study are also in coherence with the results of Krikler et al..(2000) who observed that *S. aureus* strains exhibit slight band variation in their SDS–PAGE whole-cell protein profiles, figure (7). This study obviously show that the electrophoretic method can provide valuable epidemiological information and may be used in the isolation of *Staphylococcus* strains. Some other researchers (Pereira et al ., 1995; Lopes et al., 1990; Maniatis et al., 1982; Pereira et al., 1995) obtained similar results. Our results are in agreement with the findings of Clink and Peniingtnon (Matthews et al., 1992), who reported that the protein patterns of whole cells of *S. aureus* strains and other species of staphylococci are diverse in their protein profile and have different bands.

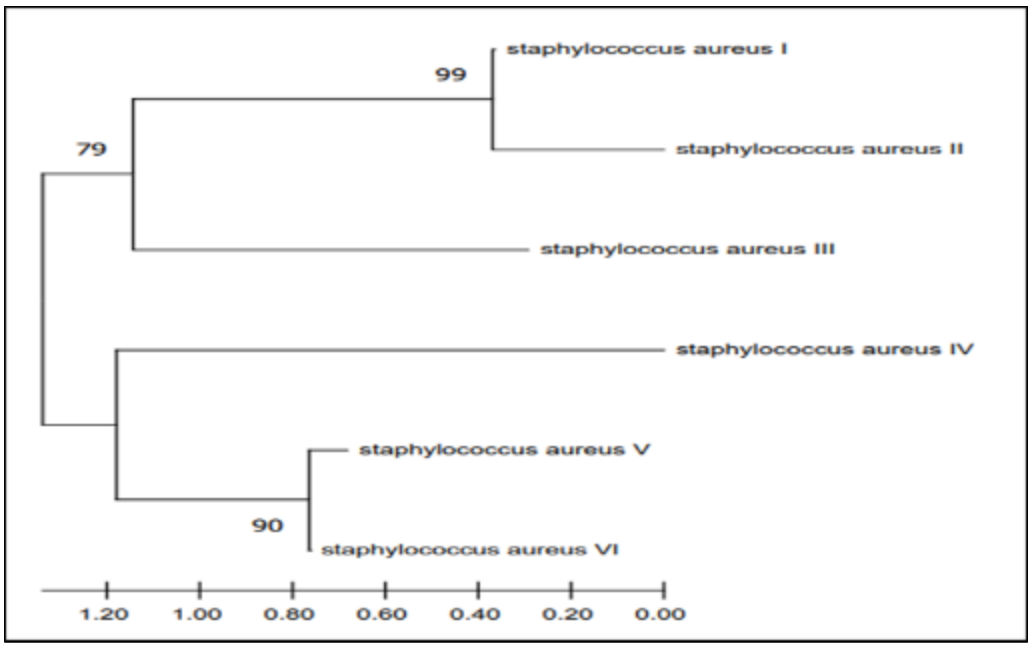

Figure 7 Dendrogram based on c cluster analysis as revealed by SDS-PAGEof *S. aureus* isolates

4. Conclusion

SDS-PAGE whole-cell protein finger printing was highly accurate in identifying clinically prevalent staphylococci species and proved to be able to detect atypical isolates. In addition, it clustered together typical and atypical isolates of each staphylococcal species.

Compliance with ethical standards

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Disclosure of conflict of interest

The Authors proclaim no conflict of interest.

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