Heliyon 5 (2019) e02231

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.heliyon.com

Molecular typing of *Staphylococcus aureus* from different sources by RAPD-PCR analysis

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ARTICLE INFO

Keywords:
Microbiology
Epidemiology
RAPD-PCR
Food
Human
Bovine mastitis
Staphylococcus aureus
Banknotes

ABSTRACT

Staphylococcus aureus is an opportunistic bacterium which is carried as a normal flora organism but has a major role in the epidemiology and pathogenesis of different staphylococcal infections in humans and animals. Fifty *S. aureus* isolated from banknotes, foods, human infections and bovine mastitis were subjected to DNA fingerprinting by randomly amplified polymorphic DNA (RAPD) analysis to examine their genotypic polymorphism and investigate the amount of genetic relatedness among these various isolates. At 100% RAPD profile similarity level, isolates were classified into four, five and seven groups of the same clone, according to the RAPDPCR with OLP6, OLP11 and OLP13 primers, respectively. Amplification of the isolates resulted in several polymorphic bands ranged from >50 to >1500 bp in size. Maximum number of bands was obtained by primer OLP13 which produced seven bands in bovine mastitis isolates. Most polymorphisms were observed in isolates of bovine mastitis and the lowest were associated with human infections isolates. There was no relationship between the RAPD patterns and the sources of isolates, except the three clusters which showed host specificity and only included the strains from the same sources. The results confirm the wide genotypic diversity of the studied *S. aureus* strains. RAPD-PCR technique can be a valuable tool for assessing the genetic relationship, detection of polymorphism in *S. aureus* and tracing the sources and management of *S. aureus* infections.

1. Introduction

Staphylococcus aureus (*S. aureus*), a facultative anaerobic gram-positive bacteria, is one of the most significant opportunistic pathogens. In humans, *S. aureus* is a major cause of foodborne-acquired and hospital-acquired infections. In animals, it elicits mastitis which contributes to major economic loss to the dairy industry (Radwan et al., 2015). It can access milk through direct excretion from udders suffering clinical and sub-clinical staphylococcal mastitis, and by environmental contamination during the milk's handling and processing (Scherrer et al., 2004; Jørgensen et al., 2005). *S. aureus* commonly harbored by about 30–60% of healthy people in the nares and skin of humans and does not always cause disease (Guidi et al., 2018).

S. aureus is involved in a wide variety of infections found in human and animals, an important cause of bovine mastitis that is one of the most cost-intensive disease in the dairy industry (Rodríguez-Calleja et al., 2006; Morandi et al., 2010; Dufour et al., 2012). Owing to zoonotic potential of *S. aureus*, control of this bacteria is not only of great economic importance in the dairy industry but also a remarkable public health concern (Kümmel et al., 2016). Some strains are responsible for human food poisoning because of the production of enterotoxins in foodstuffs. Staphylococcal enterotoxins (SEs) that cause abdominal pain, nausea, emesis, and diarrhea, are heat-stable and resistant to human gastrointestinal proteases (Dittmann et al., 2017).

Many phenotypic and genotyping methods have been used in polymorphism analysis including biotyping, multilocus sequence typing (MLST), multiocus variable number tandem repeat analysis (MLVA), pulse field gel electrophoresis (PFGE) and PCR based methods such as random amplified polymorphism DNA (RAPD) PCR, restriction fragment length polymorphic DNA (RFLP) PCR (Hennekinne et al., 2003; Bens et al., 2006; Rabello et al.,2007).

The aim of this study was to assess the genotypic polymorphism among *S. aureus* isolate were recovered from banknotes, foods, human infections and bovine mastitis milk by RAPD-PCR, and also to investigate the amount of genetic relatedness among these various isolates.

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https://doi.org/10.1016/j.heliyon.2019.e02231
Received 14 April 2019; Received in revised form 12 June 2019; Accepted 1 August 2019

2. Materials and method

2.1. Isolation and identification of S. aureus

A total of fifty *S. aureus* isolates (12 from banknotes, 12 from foods, 13 from human infections and 13 from bovine mastitis milk) were collected. Banknote isolates were randomly isolated from banknotes of banks in Shiraz, Iran and food isolates were kindly provided by Dr. T. Zahraei Salehi, College of Veterinary Medicine, Tehran University, Iran, which were isolated from restaurants in the University of Tehran. Mastitis isolates were isolated from milk samples of cows with mastitis from farms around Shiraz, Iran, and human infection isolates were collected from patients with nosocomial infections in Namazi Hospital, Shiraz, Iran. Consent form was used to get sample from each patient and the experiment was approved by the ethical committee of the School of Veterinary Medicine, Shiraz University, Shiraz, Iran.

These experiments were biochemically characterized as *S. aureus* by the conventional biochemical tests such as mannitol fermentation, hydrolysis test of gelatin and urea, protease activity on milk agar medium (Merck, Germany), lipase production on egg yolk agar medium (HiMedia, Mumbia) and hydrolysis of esculin following standard methods (Swan, 1954; Cruickshank et al., 1975). Then stored in Luria-Bertani (LB) broth (Merck, Germany) at -70 °C with 25% glycerol until use (Bair-Parker, 1974).

2.2. DNA extraction and RAPD-PCR analysis

DNA extraction was done using the CinnaPure DNA kit (CinnaGen, Iran) as described by the manufacturer. RAPD-PCR reactions were performed with oligonucleotide primers OLP6 (5′-GAGGGAAGAG-3′), OLP11 (5′-ACGATGAGCC-3′) and OLP13 (5′-ACCGCCTGCT-3′) as described by Williams et al. (1990). Amplifications were carried out in a total volume of 25 μl containing 0.75 μl of oligonucleotides primers (3 μM), 0.75 μl dNTPs (200 μM of each deoxynucleoside triphosphate) (CinnaGen, Iran), 1.5 μl of $MgCl_2$ (3.5 mM) (CinnaGen, Iran), 0.2 μl of *Taq* DNA polymerase (2.5 U) (CinnaGen, Iran), 2.5 μl of 10X PCR buffer (CinnaGen, Iran), 16.5 μl of sterile distilled water. Rather than “3μl” an estimate of the amount of DNA (ng) is needed.

Amplification of DNA fragments was performed in a MJ Mini Thermal Cycler (BIO-RAD, USA) with initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 93 °C for 1 min, annealing at 37 °C for 90 S and extension at 72 °C for 1 min, with a final extension of 7 min at 72 °C.

Amplified products were then resolved by electrophoresis in 1.5% agarose gel (CinnaGen, Iran) containing 0.5 μg/ml ethidium bromide (CinnaGen, Iran) and visualized under UV light by Gel Doc (UVitec, UK). A 100 bp DNA ladder (CinnaGen, Iran) was used as a DNA fragment size marker in all gels.

2.3. Fingerprint pattern analysis

The RAPD-PCR banding patterns generated with each primer were analyzed using GelCompar II 6.6.11 software program (Applied Maths, Belgium). Cluster analyses were carried out and dendrograms were created using the unweighted pair-group method with averaging (UPGMA). The DNA relatedness (percentage of similarity) was estimated by using the Dice similarity coefficient.

Isolates are designated genetically indistinguishable if their restriction patterns have the same numbers of bands and the corresponding bands are the same apparent size. Profiles that exhibited ≥94% Dice similarity (≤3 band differences) were considered as closely related strains or clones. Similarity of *S. aureus* isolates with 65% or greater refereed the probability of the same origin (possibly related isolates) (Tenover et al., 1995).

3. Results

In this study, genotypic polymorphism and the relatedness of fifty *S. aureus* isolates from banknotes, foods, human infections and bovine mastitis milk were characterized by RAPD-PCR analysis with three different primers (OLP6, OLP11 and OLP13). Amplification of the *S. aureus* strains with the chosen primers resulted in several polymorphic bands ranging from >50 to >1500 bp in size (Fig. 1).

RAPD analysis with primer OLP6 resulted in one to four clear bands (Fig. 1 and Table 1). At 100% RAPD profile similarity value, four major *S. aureus* clusters (RAPD profiles A-D) including genetically indistinguishable isolates were detected by OLP6 primer (Fig. 2a). Cluster A grouped only three bovine mastitis isolates. Cluster B contained six isolates (three from bovine mastitis, one from banknotes and two from foods), which were possibly related to isolates of cluster A (66.7% RAPD profile similarity). Twelve isolates were distributed in cluster C containing four from foods, two from bovine mastitis and six from banknotes. All isolates from human infections were grouped in Cluster D; this cluster contained 29 isolates (thirteen from human infections, five from bovine mastitis, five from banknotes and six from foods) (Fig. 2a).

Amplifications with primer OLP11 yielded zero to five clear bands (Fig. 1 and Table 1). OLP11 oligonucleotide discriminated the fifty *S. aureus* isolates into four clusters (A-D) of genetically indistinguishable isolates with 100% RAPD profile similarity (Fig. 2b). Cluster A contained 8 isolates (four from human infections, three from bovine mastitis and one from banknotes). Nearly half of *S. aureus* isolates (23) belonged to cluster B (six from banknotes, ten from foods and seven from bovine mastitis), which can be merged with cluster A to form a cluster containing the majority of *S. aureus* strains with possibly related genomes (66.7% similarity). Cluster C grouped 8 isolates including three bovine mastitis and five banknote isolates. Cluster D contained 9 isolates (seven from human infections and two from foods). The DNA of two isolates from human infections were not amplified by OLP11 oligonucleotide RAPD-PCR (Fig. 2b).

RAPD analysis with primer OLP13 resulted in two to seven clear bands (Fig. 1 and Table 1). Seven clusters (A-G) containing isolates with 100% RAPD profile similarity could be distinguished by primer OLP13 (Fig. 2c). All the six isolates of cluster A were from bovine mastitis samples. Cluster B only contained one isolate from human infections and cluster C only grouped the six isolates from foods. Four isolates from human infections and two isolates from bovine mastitis belonged to cluster D which had 85.7% similarity with cluster C and 75.6% similarity with cluster E which contained only one isolate from bovine mastitis (possibly related isolates). Cluster F contained 10 isolates (two from human infections, two from bovine mastitis and six from banknotes) which had 72.8% RAPD profile similarity with clusters of C-E. Fourteen isolates were grouped in cluster G (two from bovine mastitis, six from banknotes and six from foods). At 65% similarity cut-off value, the clusters of C–F can be merged together (possibly related isolates). Six isolates from human infections were not amplified by OLP13 RAPD-PCR (Fig. 2c).

4. Discussion

Staphylococcus aureus is a major opportunistic pathogen which is a leading cause of nosocomial infection in human and bovine mastitis and one of the most common agents of food-poisoning (Peton and Le Loir, 2014; Hennekinne et al., 2012; Kadariya et al., 2014; Dittmann et al., 2017). *S. aureus* has been considered as a great cause of zoonotic disease and the possible transmission amongst livestock and human through close contact, handling and use of infected food of animal origin (Kateete et al., 2013; Wang et al., 2018).

Lack of proper hygienic measures during food preparations such as handling the foods without washing hands leads to contamination of the foods by direct contact or through the respiratory secretions of food handlers who carry pathogenic *Staphylococcus*. Foods involved with

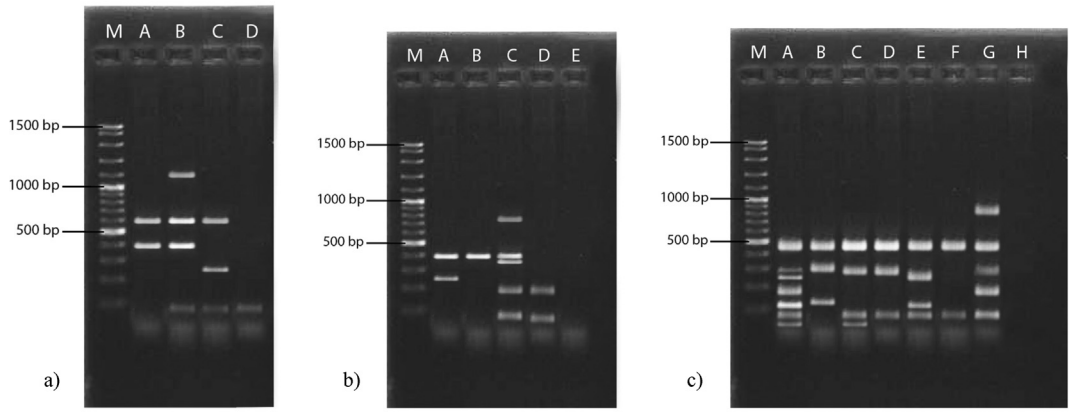

Fig. 1. RAPD-PCR patterns of *S. aureus* isolates from banknotes, foods, human infections and bovine mastitis generated with primers (a) OLP6, (b) OLP11 and (c) OLP13. M: 100 bp DNA ladder.

Table 1
Results of RAPD-PCR analysis with OLP6, OLP11 and OLP13 primers on fifty *S. aureus* isolates.

Category	Results of RAPD-PCR			Combination of 3 primers
	OLP6	OLP11	OLP13	
Number of bands				
Zero	0 (0%)	2 (4%)	6 (12%)	8/150 (5.33%)
One	29 (58%)	23 (46%)	0 (0%)	52/150 (34.66%)
Two	3 (6%)	17 (34%)	10 (20%)	30/150 (20.0%)
Three	12 (24%)	0 (0%)	7 (14%)	19/150 (12.66%)
Four	6 (12%)	0 (0%)	7 (14%)	13/150 (8.66%)
Five	0 (0%)	8 (16%)	14 (28%)	22/150 (14.66%)
Seven	0 (0%)	0 (0%)	6 (12%)	6/150 (4.0%)
Sources of isolates				
Foods	3 clusters	2 clusters	2 clusters	7/15 (46.66%)
Human infections	1 cluster	2 clusters	3 clusters	6/15 (40.0%)
Bovine mastitis	4 clusters	3 clusters	5 clusters	12/15 (80.0%)
Banknotes	3 clusters	3 clusters	2 clusters	8/15 (53.33%)

staphylococcal food poisoning are those that are made with hand contact and require no additional cooking (post-processing contamination). These foods are stored at room temperature which allows growth of *S. aureus* and production of the enterotoxins. *S. aureus* is also present in food animals and dairy cattle, sheep and goats, and could contaminate the milk of animals with clinical or subclinical mastitis (Argudin et al., 2010).

Banknotes are highly contaminated by pathogenic bacteria and because of its frequent passing from hand to hand, play a role in the transmission of *S. aureus* to other people through the hand contact. *S. aureus* are commonly isolated from banknotes from food outlets and hospitals. Simultaneous handling of food and banknotes could lead to the spread of nosocomial infections (Angelakis et al., 2014).

Typing of *S. aureus* strains may indicate feasible differences in their characteristics also it is helpful to distinguish circulation patterns of these bacteria between several hosts and sources. Many phenotypic and genotypic methods like biotyping, antibiotic susceptibility testing, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and PCR-based techniques including random amplified polymorphisms DNA (RAPD) and restriction fragment length DNA (RFLP) PCR have been used in this way. However, PFGE and MLST are effective methods for typing *S. aureus* strains, they are expensive, effortful, and technically demanding. Currently, PCR-based typing methods are effective techniques for investigations of strain origin, clonal relatedness among strains and epidemiology. Among these techniques, RAPD-PCR typing is a simple, useful and economically affordable technique which has been widely used for differentiation of *S. aureus* isolates (Tenover et al., 1994; Van der Zee et al., 1999; Deplano et al., 2000; Neela et al., 2005).

In this study, genetic polymorphism of the *S. aureus* strains became evident by the RAPD-PCR analysis (Figs. 2a-2c). At 100% similarity level which correspond to genetically indistinguishable isolates, the fifty *S. aureus* isolates from different sources were classified into four, five and eight groups of the same clone, according to the RAPD-PCR with OLP6, OLP11 and OLP13 primers, respectively.

In the RAPD patterns, some bands were common in all the samples and some were not evident. Maximum amplification was obtained by primer OLP13 which produced seven bands in six bovine mastitis isolates (Table1). Most polymorphisms and strain variability were observed in isolates of bovine mastitis (12 profiles by three primers) and the lowest were associated with human infections isolates (6 profiles by three primers). As in amplification with primer OLP6, all the human infections isolates were clustered together in one group (Fig. 2a).

Dendrograms showed that several isolates could not be distinguished from each other with these oligonucleotides (Figs. 2a-2c). On the basis of which oligonucleotides were used for RAPD-PCR, the strains that clustered together can be different hosts differed. Certain clusters (cluster A in OLP6 and cluster A, C in OLP13 RAPD-PCR) showed host specificity and only included the strains from the same sources. This means that these strains might have emerged from one clone. In addition, cluster B and E in OLP13 RAPD-PCR only included one human infection and one bovine mastitis isolate, respectively. But in other clusters no relationship was found between the RAPD patterns and the sources of isolates.

Result of this study indicates that all the fifty *S. aureus* isolates were typeable using the combination of the three tested primers in RAPD analysis. Although eight isolates from human infections were untypeable with one primer but were typeable when using another primer (Table 1 and Figs. 2a-2c). These none typeable isolates may have no specific sites in their chromosome for binding of the oligonucleotides primers.

Several studies indicated that *S. aureus* strains from different hosts were differed from each other and bovine and human isolates were rarely cross-infect (Lange et al., 1999; Larsen et al., 2000; Reinoso et al., 2004). On the other hand, transfer of *S. aureus* between humans and animals is possible. Milker's hands may have a role in the spread of *S. aureus* strains associated with bovine mastitis (Reinoso et al., 2004).

In this study, RAPD-PCR technique demonstrated to be efficient in typing and assessment of the genetic relationship of *S. aureus* isolates from different hosts. The results are in agreement with the earlier report (Reinoso et al., 2004) where, the same primers were successfully applied to assess the genetic relationship between *S. aureus* isolates from bovine and human hosts. Primers OLP6, OLP11 and OLP13 had high discriminatory power according to the Reinoso et al. (2004) study. In their study, the eighty *S. aureus* isolates were divided into eleven groups, with most of the human isolates belonging to one group and bovine isolates were distributed over the other ten groups (Reinoso et al., 2004). Fitzgerald et al. (2000) characterized the *S. aureus* isolates from bovine

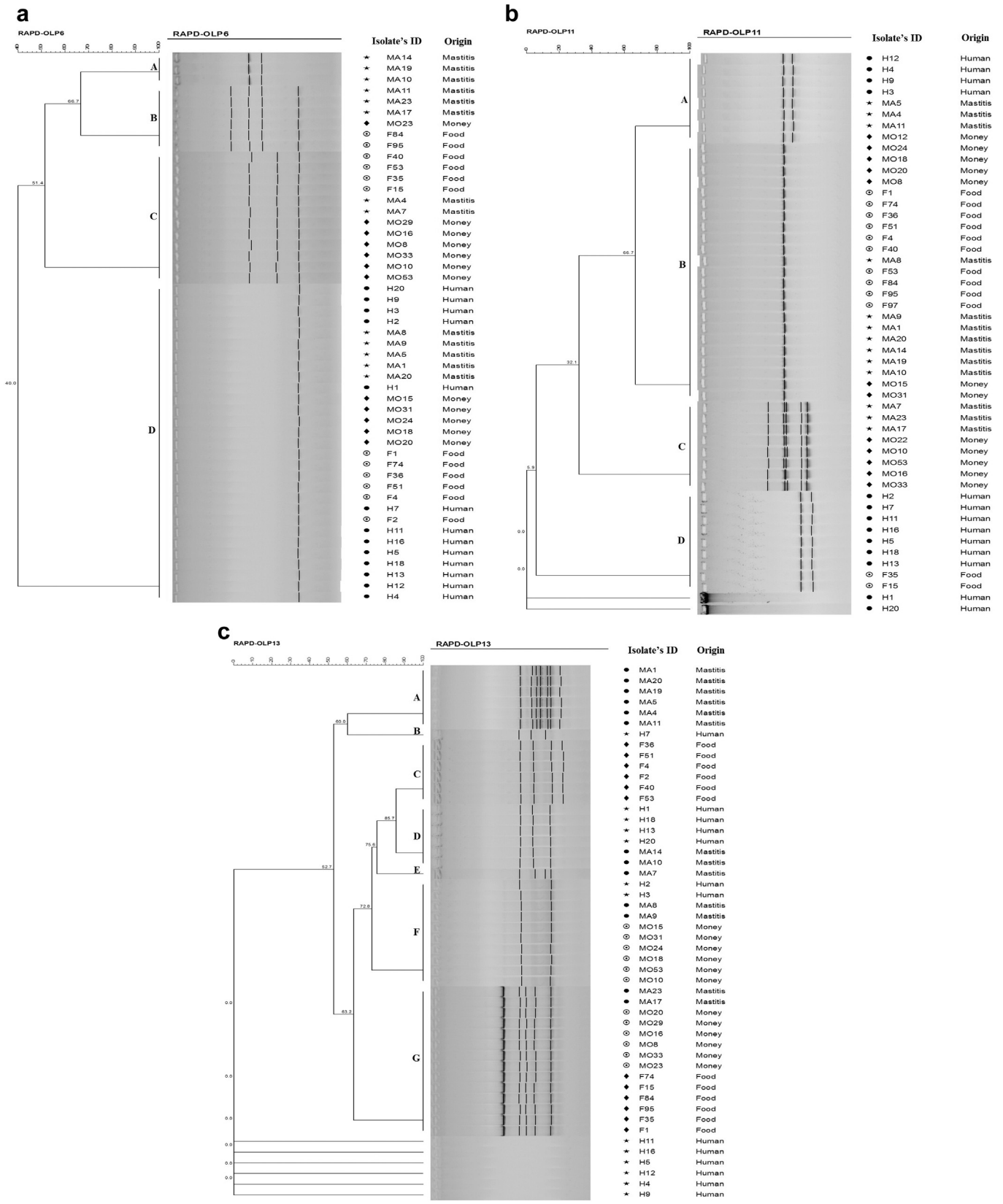

Fig. 2. a. Dendrogram of OLP6 RAPD-PCR profiles of fifty *S. aureus* isolates from banknotes, foods, human infections and bovine mastitis (based on Dice similarity coefficient). b. Dendrogram of OLP11 RAPD-PCR profiles of fifty *S. aureus* isolates from banknotes, foods, human infections and bovine mastitis (based on Dice similarity coefficient). c. Dendrogram of OLP13 RAPD-PCR profiles of fifty *S. aureus* isolates from banknotes, foods, human infections and bovine mastitis (based on Dice similarity coefficient).

intramammary infection and reported that the isolates were divided into 12 distinct clonal types on the basis of their RAPD fingerprint profiles.

Several researches have suggested that RAPD-PCR typing is widely used for *S. aureus* strains (Tambic et al., 1997; Onasanya et al., 2003; Morandi et al., 2010). Some of polymorphisms due to *S. aureus* are known but those that underscore RAPD-PCR are not so easily identified, and could be because of point mutations in and mobile genetic elements (bacteriophages, plasmids and transposons) (Lindsay, 2010; El-Jakee et al., 2010). In addition, differences in the RAPD-PCR patterns and clustering of *S. aureus* isolates among the various reports such as Fueyo et al. (2001) and Colombari et al. (2007) studies on *S. aureus* isolated from human and food samples or Pereira et al. (2002) and Reinoso et al. (2004) studies on human and bovine *S. aureus* and the current study could be due to use of different oligonucleotide primers, the source of the isolates and occurrence of mutants in *S. aureus*.

However, the three used primers generated enough polymorphic bands for distinguishing the *S. aureus* strains, but using the higher number of primers for assessing the genetic relationship between *S. aureus* isolates had increased the number of different strains.

In conclusion, the results confirm the wide genotypic diversity of *S. aureus* strains and high dissemination of certain clones through the different host's origins. RAPD-PCR analysis with combination of three primers can be useful for assessing the genetic relationship between *S. aureus* isolates from different sources. This technique can be a valuable tool for detection of polymorphism in *S. aureus* and tracing the sources and management of *S. aureus* infections, and epidemiological studies.

Declarations

Author contribution statement

Sahar Zare: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Abdollah Derakhshandeh, Masoud Haghkhah: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Zahra Naziri, Azar Motamedi Broujeni: Analyzed and interpreted the data.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors would like to thank Dr. Zahra Esmailnezhad for assisting with bacterial isolation.

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