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MOLECULAR TYPING OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM MASTITICDAIRY COWS

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ABSTRACT

The aims of this study were to isolate and identify of methicillin resistant *Staphylococcus aureus* (MRSA) from mastitic cow milk. The investigation presence of mecA genes and molecular characteristics of these isolates using eight primers of repetitive element palindromic-PCR (Rep-PCR) analysis were achieved. Ninety six milk samples, collected from clinical mastitic cows from Sharkia Governorate in Egypt, were obtained from 71cowcases. MRSA isolates were recovered by pulsed field gel electrophoresis (PFGE), staphylococcal chromosomal cassette (SCC) mec typing, staphylococcal protein A. A total of 11 MRSA isolates were identified. All strains were showed a positive mecAI genes band (with size about 162 bp long) and mecAII gene band (with size about 449 bp long) and negative in mecAIII gene band (with size about of 540 bp long). The results of Rep-PCR analysis for the 11 MRSA isolates revealed approximately 114 different banding pattern, 51 of them consider as monomorphic bands with a percentage about 44.8%, and other 63 fragments consider as polymorphic bands with about 55.2%. The dendogram based on banding patterns dividing the 11 isolates into two

clusters with about 67% genetic similarities. Our results indicate that the presence of sufficient nuclear DNA level variations among the studied MRSAisolates using Rep-PCR analysis, therefore, the Rep-PCR data might be a good source of information about the diversity of native *S. aureus* isolates.

KEYWORDS: The mastitis cow milk; Rep-PCR; mecA genes; *Staphylococcus aureus.*

INTRODUCTION

Bovine mastitis is one of the most costly and complex diseases of the dairy industry. The complexity is reflected in the numerous causative pathogens, the variety and magnitude of the physiological responses to these pathogens and the variation in efficacy of control measures for different causative organisms (Claudia et al., 2016). Mastitis, inflammation of the mammary gland with local and or general symptoms that occasionally result in a systemic infection, can be caused by a wide range of micro-organisms, including gram-negative and gram-positive bacteria (Le Marechal et al., 2011). *Staphylococcus aureus*is the most common etiological pathogen of contagious bovine mastitis and it has the potential to develop resistance to almost all the antimicrobial agents (Wang et al., 2015). *Staphylococcus aureus* is one of the predominant causative agents of clinical mastitis in most countries (Pereira et al., 2011) including Egypt (Ibrahim and El-Gedawy, 2013; El-Behiry et al., 2015). The current conservative practice of treating mastitis with antibiotics is not only economically unviable in the long term, especially for the marginal and small-scale dairy farmers in Egypt, but also promotes the emergence of strains resistant to antibiotics, including methicillin (Haran et al., 2012; Paterson et al., 2013). Given the recent reports that even MSSA (methicillin-sensitive *S. aureus*) can become resistant to certain antimicrobials when it is present as a biofilm, treatment of bovine mastitis caused by MSSA becomes problematic (Espeche et al., 2012; Babra et al., 2013). Inaddition, there is increasing evidence of cross-transmission of virulent *S. aureus* between dairy cattle and humans (Sakwinska et al., 2011; Fluit, 2012), with implications for public health. There is an urgent need for a suitable vaccine against mastitis to reduce both the bacterial load and the probability of successful cross-transmission. During the past decade, the epidemiology of *S. aureus* mastitis in dairy cattle has been studied using various molecular typing methods. Techniques that rely on the comparison of electrophoretic patterns, such as Pulsed-Field Gel Electrophoresis (PFGE) (Annemuller et al., 1999; Jørgensen et al., 2005), the genetic variability and relationships among the isolates were established by random amplified polymorphic DNA(RAPD)-PCR analysis (Salgado-Ruiz et al., 2015). Repetitive element

palindromic-PCR (Rep-PCR) has been identified as a simple PCR-based technique with the following characteristics: (i) low cost, (ii) high discriminatory power, (iii) suitable for a high-throughput of strains, and (iv) considered to be a trusty tool for classifying and typing a wide range of Gram-negative and several Gram positive bacteria(Abdollahi et al., 2016).

This study was conducted to investigate the molecular characterization of *S. aureus* isolated from dairy cow mastitis in Sharkia Governorate in Egypt. The presence of the resistance was evaluated in *S. aureus*. Staphylococci antimicrobial resistance was performed by detection of *mecA* genes and biodiversity of *S. aureus* by repetitive sequence polymerase chain reaction (Rep-PCR).

MATERIALS AND METHODS

Clinical examination of udder and milk

Udder inspected and must be free from any abnormal lesions or anatomical malformation. Milk was examined for consistency, color and visible abnormalities. Clinical mastitis was recognized by abnormal milk and signs of udder infection (Kedir et al., 2016).

Bacteria isolation

95 milk samples were collected from mastitic cows in special veterinary clinic in Skarkia governorate in Egypt. Before milk sampling, the teats were disinfected with cotton moistened with 70% alcohol. After discarding the first few squirts of milk about 20 ml were collected in sterile universal bottles and kept in an icebox, and transported immediately to the laboratory for bacteriological analysis according to (Suleiman, *et al*., 2012).

Isolation and identification of bacteria

Bacteriological examinations were carried out following standard methods (Quinn *et al*., 1994). Briefly, a loopful of each milk sample was streaked on 7% sheep blood agar (Merck). MacConkey agar (Merck) plates were also used in parallel to detect *Enterococcus* species and any Gram-negative bacteria. Inoculated plates were incubated aerobically at 37°C for 24-48 hrs. Presumptive identification of bacterial isolates was made based on colony morphologic features, Gram-stain reaction, hemolytic characteristics and a catalase test. Staphylococci and Micrococci were identified based on their growth characteristics on mannitol salt agar, coagulase production by using Staphylect plus reagent (Oxoid), catalase and oxidase tests. Isolates identified tentatively as *Streptococci* were evaluated according to CAMP reaction, growth characteristics on Edward's medium (Oxoid), hydrolysis of esculin and sodium

hippurate, catalase production, and sugar fermentation tests. Gram-negative isolates were sub-cultured on Mac Conkey agar for competitive flora and further tested using triple sugar iron (TSI) agar (Merck), the IMViC test (indole, methyl red, Voges-Proskauer and citrate utilization tests), urea, lysine and ornithine decarboxylase and oxidase reactions.

Oxacillin Screening Agar test

Test was performed according to Jain *et al*. (2008) with some modification. Briefly, plates were prepared with Mueller–Hinton agar supplemented with 4% (w/v) NaCl containing oxacillin at a concentration of 6µg ml^{-1} (OSA 6µg ml^{-1}). The plates were spot inoculated with a cotton swab dipped into a 0.5 McFarland standard suspension of each isolate, Oxacillin resistance was confirmed by bacterial growth after 24 h incubation at 35ºC.

***Staphylococcus aureus* culture for DNA extraction**

According to Stephens (2008), bacterial growth for the purpose of DNA extraction was prepared as follows: 20 µl of stock solution was streaked onto a Brain Heart Infusion (BHI) agar plate (prepared as specified, Oxoid Australia Pty Ltd, Adelaide) and cultured overnight at 37ºC. the following day a single colony was selected and suspended in a 5 ml falcon tube (Becton Dickinson, New South Wales, Australia) containing BHI broth (prepared as specific, Oxoid Australia Pty Ltd, Adelaide) and cultured overnight at 37ºC, with shaking.

Genomic DNA extraction

The cell pellets from all isolates were used to extract genomic DNA using (Jena Bioscience, Germany) extraction kit following the manufacturer's instructions.

Detection of antibiotic resistance genes

PCR amplification for detection the three antibiotic genes mecA genes were carried out according to Al-Khulaifi et al., (2009). PCR amplification of mec A genes was carried out using the following primers.

Primers name	Forward sequence 5`→3`	Reverse sequence 5`→3`
mec A I	TCCAGATTACAACTTCACCAG	CAATTCATATCTTGTACCG`
mec A II	CTCAGGTACTGCTATCCACC	CACTTGGTATATCTTCACC
mec A III	ATCGATGGTAAAGGTTGGC	AGTTCTGCAGTACCGGATTTCC
mec SCC I	GCTTTAAAGAGTGTCGTTACAGG	GTTCTCTCATAGTATGACGTCC
mec SCC II	CGTTGAAGATGATGAAGCG	CGAAATCAATGGTTAATGGACC
mec SCC III	CCATATTGTGTACGATGCG	CCTTAGTTGTCGTAACAGATCG

PCR mixtures (25 µL) contained 1 µL of DNA template, 12.5 µL master mix (Promega) and 10pM of each primer and 9.5 µL sterilized distilled water. PCR amplifications were performed in C1000^{TM} Thermo Cycler Bio-Radusing the following program for mec A: initial denaturation at 95°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s of annealing at 54°C, and 1 min of extension at 72°C, with a final extension of 7 min at 72°C. For mecScc, amplification was carried out with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 1 min at 95°C, 1 min of annealing at 58°C, and 1 min of extension at 72°C, with a final extension of 10 min at 72°C. PCR products were run on 1.5% agarose gels, stained with ethidium bromide and visualized by UV illumination and were photographed by a Bio-Rad Gel Doc 2000 device.

Rep-PCR analysis

For repetitive sequence analysis, PCR conditions for *Staphylococcus* isolates in the present investigations were standardized. Eight repetitive sequence primers (BOX-A1, $(GTG)_5$, ERIC-I, Rep-1, Rep-2, Rep-12, Rep-18 and Rep-19) were used to amplify genomic DNA of the *S. aureus*isolates according to (Hassan et al.a,b, 2014; Gaber et al., 2015). Following the experiments for optimization of component concentrations, PCR amplification of repetitive sequence primers were carried out in 25 µl volume containing 1 µl (20 ng) of genomic DNA, 12.5 µl of Go Taq® Green Master Mix, Promega, USA. 1 µl of primer (20 p.mol), deionizerD distilled water (up to a total volume of 25 µl). For DNA amplification, the C1000TM Thermo Cycler Bio-Rad, Germany, was programmed under the conditions involving denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 45 Sec and primer extension at 72°C for 2.5 min; final extension step at 72°C for 10 min.

Data analysis

In order to determine the genetic relationship among studied bacteria, Rep-PCR data were scored for presence (1) or absence (0) of the bands using Gene Tools software from Syngene. A simple matching coefficient was estimated by means of the Jaccard's coefficient to construct a similarity matrix. Cluster analysis and dendrogram were produced on the basis of the unweighted average pair group method (UPGMA) using the NTSYS-PC Statistical Package (Rohlf, 2000).

RESULTS AND DISCUSSION

Mastitis, defined as acute or the chronic inflammation of the mammary gland, affects lactating animals including cattle, goats, sheep, buffaloes and camels. The disease is

considered to be one of the most important causes of economic losses in the dairy industry worldwide (Karimuribo *et al*., 2005). The collected milk samples (95 samples) were examined for detection of bacterial causes of mastitis; bacteria were isolated from 70 cases of mastitis. Out of the 70 cases. *S. aureus* was isolated from 25 cases with percentage 35.7, including 10 isolates were resistant to methicillin with percentage 14.3, while 15 isolates were sensitive to methicillin with percentage 21.4 (Table 1). Both types were differentiated by the Oxacillin Screening Agar test. From other hand, *E. coli* was isolated as single cause of mastitis from 20 of 70 cases with percentage 28.6, while as mixed infection with Streptococci was isolated from 10 of 70 cases with percentage 14.3 (Table 1). This mean that *E. coli* isolate as single or mixed was the predominant infection in mastitic animals at Sharkia Governorate. This result was in agreement with that of Smith and Hogan (2008). *Pseudomonas arigenosa* were isolated from 5 of 70 cases with percentage 7.1. *Streptococci agalactia* isolated as single infection from 10 of 70 cases with percentage 14.3 and as mixed infection with *E. coli* from 10 of 70 cases with percentage 14.3 (Table 1). Streptococci form a large group of organisms which are associated with bovine udder infections (Ibrahim and El-Gedawy, 2013). The most common pathogen cause bovine mastitis is *S. agalactiae.* This is a highly infectious pathogen that can rapidly spread among a herd from a single infected animal. In the current study, *S. agalactiae* was isolated with a percentage of 20. This results was in agree with Ibrahim and El-Gedawy, (2013), who reported that mastitis caused by *S. agalactiae* with percentage of 19.7

Detection of antibiotic resistance genes for *S. aureus*

From literatures, *Staphylococcus aureus* is the most important mammary gland pathogens responsible for bovine mastitis (Alharthi, 2016). Despite implanting intensive control measures, it is difficult to eradicate the intramammary infections caused by this pathogen and remains a substantial economic problem. The cause of this problem may be due to *Staphylococcus aureus* produces a broad spectrum of surface components (proteins and capsular polysaccharide) and exotoxins, which are the virulence factors involved in the pathogenesis of bovine mastitis as these toxins and products are injurious to milk producing cells of the mammary gland, impair glands and immune defense mechanisms, while they are capable to reside intracellular contributes the ability of *S. aureus* to establish a chronic infection that can persist for the life of the animal Taverna *et al*.(2007).

In the present study, a total of 11 strains of *S. aureus* were additionally examined for different virulence genes. Antimicrobial resistance of *S. aureus* especially methicillin resistant *S. aureus* (MRSA) continues to be a problem for clinicians worldwide Hassan et al., (2014a).To determine susceptibility to methicillin, following NCCLS's recommendations, oxacillin was used, as it is more stable than methicillin in the lab conditions and is able to recognize cross resistance. In addition, agar screen method was used which is preferable to the disk diffusion method NCCL (2000).PCR amplification of the gene segment encoding the *mecAI* revealed a size of approximately 162 bp of 11 isolates (Table 2 and figure 1a). The amplification of the gene segments encoding the *mecAII* revealed the typical size approximately 449 bp of 11 isolates (Table 2 and figure 1b). Moreover, *S. aureus* coding the *mecAIII* is considered an important gene for typing of *S. aureus*; it was determined in this study in the majority of isolates. As can be noticed in Table 2, PCR amplification of this gene revealed that no specimens contained the *mecAIII* gene with size about 540 bp. From the previous results, various virulence genes were detected in the *S. aureus* strains isolated from bovine clinical and subclinical mastitis milk in Egypt and Kingdom of Saudi Arabia (Hanssen et al., 2006; Hassan et al., 2014a). The *mecA* genes are responsible for the resistance of *Staphylococcus* to the β-lactams antibiotic group (Zhang et al., 2009). This genetic material is not belonging to the *Staphylococcus* genome but it has been acquired by *Staphylococcus spp.*The control of bovine mastitis is very important not only in the Middle East but also worldwide as mastitis is considered the main cause among several diseases involved in reduction of milk production (Hussain et al., 2012; Khan et al., 2013). Consequently, it is crucial to examine the mastitis causing bacteria using molecular methods as forceful tools to control IMI. Because *S. aureus* is the most commonly contagious mastitis pathogens worldwide, it is important to reveal subtypes and virulence factors of these agents to develop effective control strategies against mastitis caused by both pathogens (Khan et al., 2013; El-Behiry et al., 2015). In addition, an effective vaccine against IMI is not available, therefore prevention and control of mastitis needs detection of the principal antigenic determinants for the strategy and progress of more proficient vaccines against mastitis causing bacteria especially *S. aureus* (El-Behiry et al., 2015).

Our results confirm the conclusion of Ibrahim and El-Gedawy, (2013), which PCR could detect the *mecA* gene in all 11 strains (100%). Similar results obtained by Amin *et al*. (2011) who reported that all tested mastitic milk clinically were positive for culture and multiplex PCR designed for detection of Triple micro-organism *S. aureus, E. coli* and *Streptococcus*

agalactiae. Also, the author stated that, when compared PCR with culture, the PCR assays are less time consuming. The accuracy of PCR may be attributed to the fact that PCR detects living and dead organisms, since it is based on detection of organism DNA, while culture detects only living organisms. PCR assays could detect fewer numbers of organisms per milliliter of milk than could be detected by direct culture. Also, Manakant and Tanaya, (2012), found that 23 isolates of *S .aureus* recovered from milk samples were identified phenotypically and genotypically as resistant to methicillin. And The *mec*A gene was confirmed in all of these MRSA isolates. In contrast to the present results. Amin *et al*. (2011) reported that *S. aureus* strains originated from bovine mastitis were identified phenotypically by some conventional tests and also were characterized genotypically by a duplex PCR targeting 16srRNA and *mec*A gene. He explained the results as conventional susceptibility tests such as agar disc diffusion and broth dilution methods may not give reliable results in detecting MRSA because of heterogenic expression of resistance. Also, he classified the *S. aureus* lack *mec*A gene and resist oxacillin as false resistant by the oxacillin disc diffusion method and he considered that it may due to another resistance mechanism such as hyperproduction of beta-lactamase. The discrepancy between results may be attributed to the differences in method of methicillin resistance detection, we use oxacillin screening agar test which more efficacy in detection of resistance. It was concluded that, the duplex PCR have the same sensitivity and accuracy as isolation and identification of *S. aureus* methicillin resistance, but with less labor and very little time in comparing with the traditional method of isolation and identification. While the PCR is more expensive than traditional methods, but rapid diagnosis of MRSA in mastitic milk help in rapid treatment and rapid cure which means money save.

Rep-PCR analysis

Rep-PCR is a new typing method that differentiates microbes by using primers complementary to interspersed repetitive consensus sequences that enable amplification of diverse-sized DNA fragments consisting of sequences between the repetitive elements (Healy et al., 2004). The Rep-PCR fingerprinting method, utilizing repetitive sequence oligonucleotides, is particularly a powerful tool for genetic studies and it is useful as a screening genotyping method (Healy et al., 2004). Rep-PCR can generate various fingerprint patterns with unlimited number of fragments (Hassan et al., 2014b). In the present study, eight Rep-PCR primers were used for estimating of genetic diversity of *Staphylococcus* isolates. Rep-PCR reactions were performed in duplicate and all amplification products were

found to be reproducible (Fig. 3 and Table 3). The Rep-PCR results using primer $(GTG)_5$ has showed a total of 17 bands sized ranged from 150-2800 bp long in all eleven *Staphylococcus* isolates. nine common bands were observed in all isolates which exhibited about 53 % monomorphism, while the other eight fragments have showed 47% polymorphism (Table 3). In case of (Box-A1) primer, a total of fourteen fragments have showed 50% polymorphism among the eleven *Staphylococcus* isolates (Figure 3). The molecular size of the amplicon products ranged from 150-2300 bp long. Also, this primer recognized different unique fragments at 2200 bp specific to isolate S4 and S10, respectively. The eight Rep-PCR primers preduced about 114 fragments, 51 of them consider as monomorphic bands with about 44.8%, and other 63 fragments consider as polymorphic bands with about 55.2%. According to genetic similarity and intraspecies differentiation, the eleven *Staphylococcus* isolates were grouped into two main different clusters with about 67% genetic similarity. S8 isolate was found to be alone in the first cluster. while, the second cluster was divided to two sub-clusters. The firist sub-cluster contained S4 and S10 only, and S1, S2, S3, S5, S6, S7, S9 and S11were grouped in the second sub-cluster. (Figure 3). Rep-PCR technique was proved to be useful genetic markers used for fingerprintingof *S. aurius* strains isolated from clinical and subclinical cases of bovine mastitis. Although major bands from Rep-PCR reactions are highly reproducible, minor bands can difficult to repeat due to repetitive sequence priming nature of this PCR reaction and potential confounding effects associated with co-migration with other markers (Patrizia and Paola, 2003; Manakant and Tanaya, 2012; Hassan and Belal, 2016). Because AP-PCR has its limitations for widespread use, another more reproducible PCR method should be considered. The Rep-PCR has been described for the molecular genotyping of *S. aureus* (Alharthi, et al., 2016). It also generates strain-specific DNA fragments when *S. aureus* DNA is used as an amplification template (Hassan et al., 2014b: Gaber et al., 2015). Therefore, the use of multiple primer sets in Rep-PCR analysis can be used as a rapid method for preliminary biotyping of *S. aurius* strains.

Table (1): Occurrence of some pathogenic bacteria in mastitic animals in Sharkia governorate.

Name	Number	Percentage	Percentage of bacterial type from total isolates
S. aureus methicillin resistant	11	11	14.3
S. aureus methicillin sensitive	15	15.6	21.4
E. coli	20	21	28.6
Pseudomonas arigenosa	5	5	7.1
Streptococcus agalactia	10	11	14.3
E. coli + Streptococci	10	11	14.3
No bacterial cause	25	26	-
Total	96	100	100

Table (2): Detection of certain virulence genes in the *Staphylococcus aureus* strains isolated from clinical and subclinical cases of bovine mastitis.

Genes	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
mecAI	+	+	+	+	+	+	+	+	+	+	+
mecAII	+	+	+	+	+	+	+	+	+	+	+
mecAIII	-	-	-	-	-	-	-	-	-	-	-

Table (3): Polymorphic bands of Rep-PCR primers and percentage of polymorphism in *Staphylococcus aurius* strains isolated from clinical and subclinical cases of bovine mastitis.

Primers	Total Bands	No. of Monomorphic Bands	No. Polymorphic Bands	% Monomorphic bands	% Polymorphic bands
BOX-A1	14	7	7	50	40
$(GTG)_5$	17	9	8	53	47
ERIC-I	13	1	12	7.7	92.3
Rep-1	13	7	6	53.8	46.2
Rep-2	14	5	9	35.7	64.3
Rep-12	13	7	6	53.8	46.2
Rep-18	15	7	8	46.7	53.3
Rep-19	15	8	7	53.3	46.7
Total	114	51	63	44.8	55.2

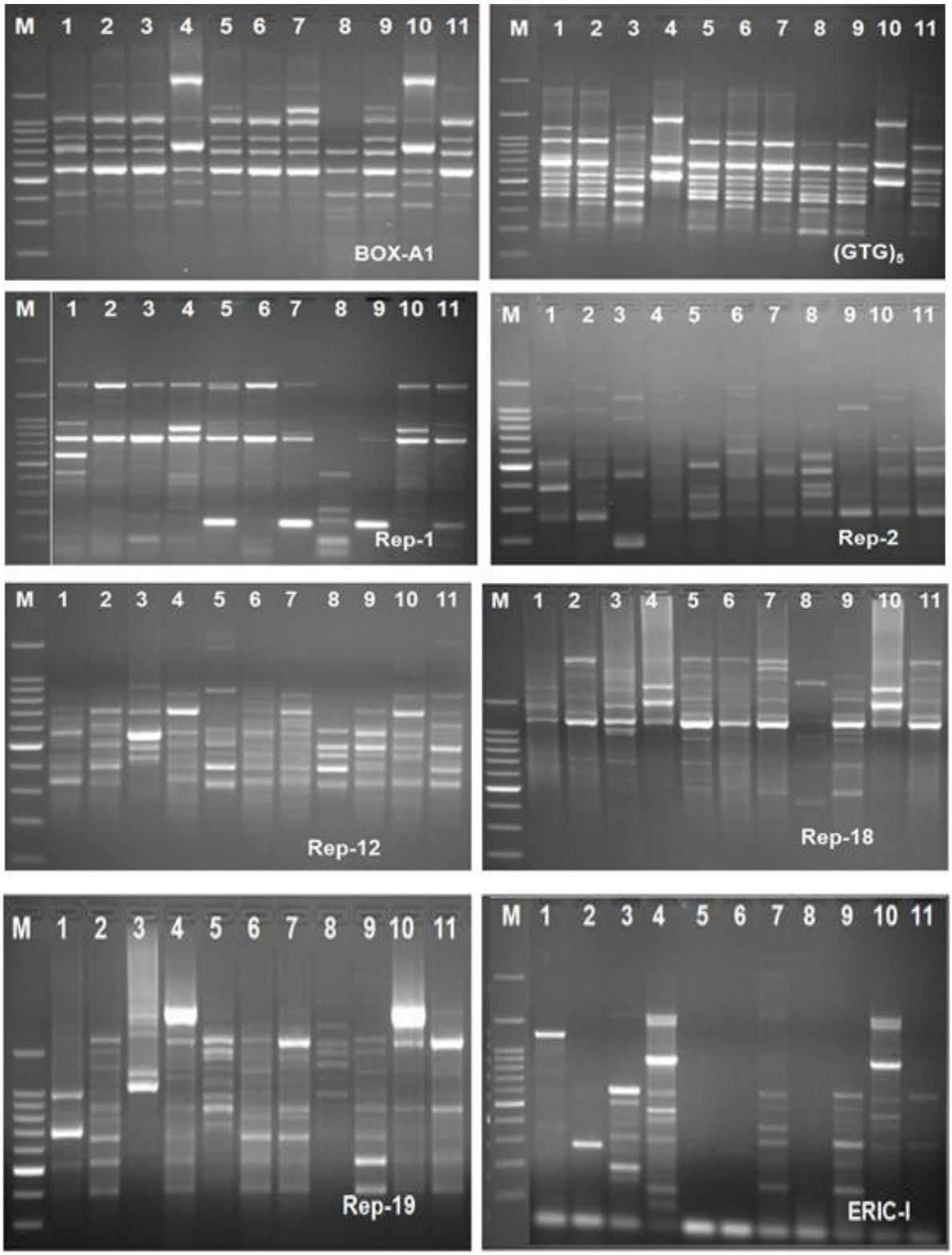

Figure (1): Rep-PCR profile of 11 *Staphylococcus* isolates generated with 8 repetitive sequence primers. First lane on each panel is 100 bp molecular weight markers.

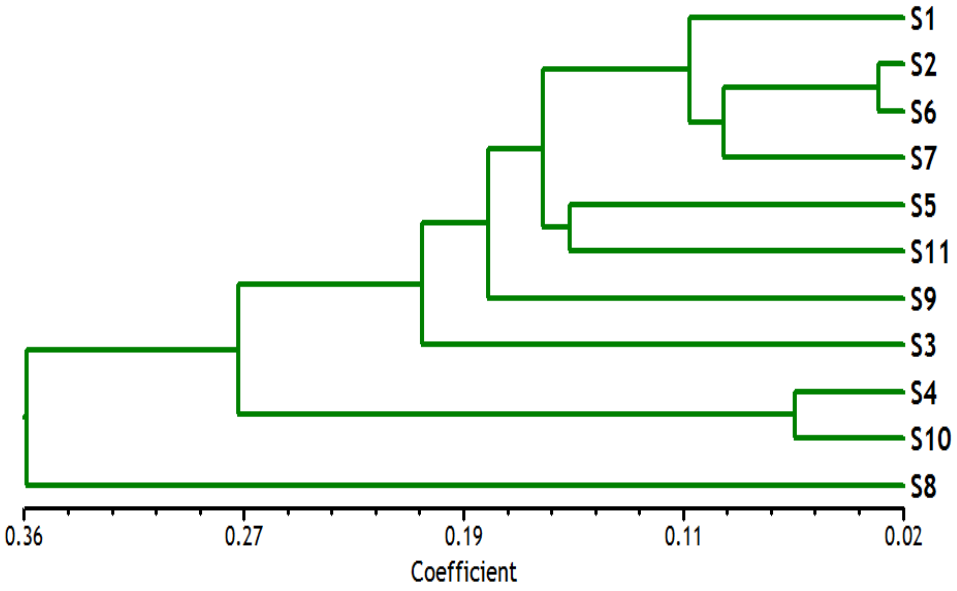

Figure (2): Dendrogram analysis among the 11 *Staphylococcus* isolates based on the 8 repetitive sequence primers.

CONCLUSION

We conclude that rep-PCR typing may be suitable for widespread use in the clinical microbiology laboratory for epidemiological typing of *S. aureus* strains. Furthermore, rep-PCR patterns are suitable for analysis with Gel Compar software and storage, provided that (i) PCR is performed with isolated chromosomal DNA and not with lysed cells and (ii) the gel electrophoresis conditions are standardized. Stored rep-PCR patterns and interlaboratory exchange of digitized fingerprints by electronic mail can play an important role in the analysis of future nosocomial MRSA outbreaks and in the monitoring of the international spread of strains with high epidemic potentials.

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