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Use of the Different Polyacrylamide Gel Electrophoresis (PAGE) Methods in the Characterization of Human Pathogenic *Staphylococcus aureus* Strains

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In the study, a total of 41 strains of *Staphylococcus aureus* including 38 clinical isolates and 3 reference strains were characterized according to biotyping, plasmid profiles and the numerical analysis of the protein profiles. The antibiogram results showed that the isolates were resistant against the tested antibiotics, except for vancomycin. Furthermore, plasmid profile analysis confirmed that the strains consisted of only one plasmid. Present findings indicated that the whole-cell and extracellular protein profiles obtained by using SDS-PAGE methods to be good typing tool for the differentiation of *S. aureus* strains at the species level, not strain level. However, Native-PAGE of whole-cell proteins was the most reliable and rapid method for differentiation between MRSA and ordinary *S. aureus* strains comparing to other applying PAGE techniques. In addition, it was determined that the same clone was responsible for most cases of MRSA and other *S. aureus* epidemic among surveyed hospitals. In conclusion, this study supposed that together application at least two different polyacrylamide gel electrophoresis (PAGE) techniques can be offer accurate and an effective approach to the investigation of taxonomic relationships within human pathogenic *S. aureus* strains.

Key Words: Nosocomial infections, *Staphylococcus aureus*, PAGE, protein profiles.

INTRODUCTION

Staphylococcus aureus is the most prevalent human pathogen that causes a number of infections in humans ranging from localized skin suppuration to life-threatening septicemia, toxic shock syndrome or food poisoning[1-4]. Although the conventional phenotypic methods based on bacterial properties have provided the standard means of differentiating *S. aureus*. These methods can be occasionally

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lead to misclassification[5,6]. For this reason, in addition to biochemical and physiological typing methods, several molecular methods have been developed for characterization of *S. aureus* strains. Among these, pulsed field gel electrophoresis (PFGE)[7-9], nested PCR assay[10], multiplex PCR analysis[11,12], PCR-based ribotyping[13], randomly amplified polymorphic DNA (RAPD)-PCR analysis[14] and ribosomal DNA polymorphism (rDNA)[15] have been used for identification and characterization of pathogenic *S. aureus* strains isolated from clinical specimens and other sources. Analysis of genetic background using by molecular typing methods is important in the sense that specific DNA characteristics may be correlated with important phenomena such as strain epidemicity and virulence. However, the most of methods are fairly laborious and the DNA restriction patterns may be difficult to interpret. Moreover, interlaboratory standardization conditions are still problematic[15,16].

A number of researchers have been employed successfully sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins in the characterization of various microorganisms[17-21]. In several cases, one-dimensional electrophenograms of whole-cell proteins and DNA-DNA hybridization data were described as having equal discriminatory capacities[19,22,23]. SDS-PAGE of whole-cell and extracellular proteins have been used for typing of *Staphylococci* isolated from clinical samples and other origins[17,19,20,24]. Nevertheless, less number of papers published regarding Native-PAGE typing of *Staphylococcal* and some bacteria by using whole-cell and extracellular proteins. The authors suggested that Native-PAGE of whole-cell and extracellular proteins was reliable for analysis of *S. aureus* isolates and *Bacillus* species; however, the differences were insufficient for differentiation of both species[25,26]. The aim of this study was not only to compare the numerical analysis results of the protein profiles of 41 *S. aureus* strains using two types polyacrylamide gel electrophoresis (PAGE) techniques but also to investigation of taxonomic relationships among them.

EXPERIMENTAL

Collection, isolation and identification of *S. aureus* strains: In the study, a total of 38 *S. aureus* strains isolated from different clinical specimens from Van (Turkey) and 3 reference strains were identified (Table-1). The clinical isolates were identified on the basis of colony morphology and biochemical properties[27-29]. The following phenotypic tests were performed: Gram stain, catalase and oxidase test; Voges-Proskauer test; degradation of starch, urease production test; utilization of glucose, mannitol, maltose, sucrose, mannose, raffinose, lactose, xylose, trehalose, ribose, fructose and galactose; anaerobic fermentation of mannitol and glucose; β-lactamase and coagulase production test, egg-yolk lecithinase; utilization of gelatine; DNase test; adherans test; growth in NaCl 10 %, growth in mannitol salt agar; hemolysis test; bacitracine sensitivity test and on MSA supplemented with 1 (mcg) of oxacillin for selective isolation of MRSA.

TABLE-1
SAMPLES OF *S. aureus* COLLECTED FROM VARIOUS BODY SITES

Strains	Specimen	Origin
ND-2, ND-4, ND-5, ND-22, ND-24, ND-29 ND-32	Wound	Van State Hospital, Turkish Ministry of Health, Van, Turkey
NA-1, NA-6, NA-16, NA-21, NA-33, NA-36	Wound	Department of Clinical Microbiology, Faculty of Medicine, Yuzuncu Yil University, Van, Turkey
NA-7, NA-11, NA-14, NA-18, NA-23, NA-25, NA-27, NA-28, NA-30	Thorax	Department of Clinical Microbiology, Faculty of Medicine, Yuzuncu Yil University, Van, Turkey
ND-3	Thorax	Van State Hospital, Turkish Ministry of Health, Van, Turkey
NA-8, NA-13	Articulation fluid	Department of Clinical Microbiology, Faculty of Medicine, Yuzuncu Yil University, Van
NA-9, NA-10	Blood	Ipek Yolu Research Hospital, Turkish Ministry of Health, 65080 Van, Turkey
NA-12	Urine	Department of Clinical Microbiology, Faculty of Medicine, Yuzuncu Yil University, Van, Turkey
ND-15	Nasal fluid	Van State Hospital, Turkish Ministry of Health, Van, Turkey
NE-17, NE-19, NE-35	Urine	Private Etfal Children Hospital, Van
NA-20, NA-31	Catheter	Department of Clinical Microbiology, Faculty of Medicine, Yuzuncu Yil University, Van, Turkey
ND-26	Drainage	Van State Hospital, Turkish Ministry of Health, Van, Turkey
NA-34	Vaginal fluid	Department of Clinical Microbiology, Faculty of Medicine, Yuzuncu Yil University, Van, Turkey
NA-37	Drainage	Department of Clinical Microbiology, Faculty of Medicine, Yuzuncu Yil University, Van, Turkey
NA-38	Sperm	Department of Clinical Microbiology, Faculty of Medicine, Yuzuncu Yil University, Van, Turkey
S. aureus RSHMB	Reference	Refik Saydam Hifsisihha Institute, Ankara, Turkey
S. aureus ATCC 6538	Reference	American Types Cultural Collection, USA
S. aureus ATCC 25923	Reference	American Types Cultural Collection, USA

Antibiograms: Antimicrobial susceptibility of isolates was done according to National Committee for Clinical Laboratory Standards (NCCLS) by disc diffusion method[30]. The following antimicrobial agents were used: vancomycin (30 mcg), ciprofloxacine (5 mcg), sulbactam (10 mcg)/ampicillin (10 mcg), penicillin (1 unit), amoxicillin (20 mcg)/clavulanic acid (10 mcg), ampicillin (10 mcg), netilmicin (30 mcg), cefotaxime (30 mcg), chloramphenicol (30 mcg), ceftazidime (30 mcg), trimethoprim (1.25 mcg)/sulfamethoxazole (23.75 mcg), piperaciline/tazobactam (110 mcg), amikacin (30 mcg), gentamicin (10 mcg), erythromycin (15 mcg), norfloxacin (10 mcg) and oxacillin (1 mcg).

Plasmid DNA extraction and agarose gel electrophoresis: Plasmid was extracted from each strain following some minor modifications of the Zhou *et al.*[31]

procedure. A 30 mL culture of all *S. aureus* strains were grown overnight on a rotary shaker at 37 °C in brain heart infusion broth (Difco, Detroit). The cells were harvested by centrifugation and washed twice in 10 mL of TES buffer (30 mM tris-hydrochloride pH 8.0, 50 mM NaCl, 5 mM EDTA). The cell pellets were resuspended in 100 µL of lysis buffer (50 mM glucose, 10 mM EDTA and 25 mM tris HCl, pH 8.0) containing 2 µL of stock lysostaphin (100 mg/L). This sample was then incubated at 37 °C for 1 h with gentle agitation followed by the addition of 200 µL of alkaline sodium dodecyl sulphate (1 % SDS in 0.2 M NaOH) and the mixture was incubated for additional 10 min at 37 °C. After 10 min, the lysates were neutralized by the addition 150 µL of 5 M sodium acetate, pH 4.8 and mixed. The final mixture was chilled on ice for 30 min and centrifuged for 10 min at room temperature. A 400 µL of supernatant was removed and transferred to 1.8 mL Eppendorf tube. Phenol-chloroform (1:1, v/v) extraction was carried out on all the samples twice. The final upper aqueous phase was then precipitated with equal volume of 100 % ethanol at -70 °C overnight. Ethanol was decanted and the DNA pellet was dried at room temperature for 2 h. The extracted DNA was dissolved with TE buffer (10 mM tris HCl pH 8.0, 1 mM EDTA, RNase 25 mg/mL). The plasmid DNA subjected to electrophoresis at 100 V, 2 h on a 0.8 % agarose horizontal slab gel unit (Midicell® Primo Submarine Gel System, USA) and stained with ethidium bromide for photography under UV illuminator (Cedex, France). Plasmid DNA molecular sizes was calculated on the basis comparison with the following standards (1 kb DNA Ladder SM0311, Fermentas).

Extraction of whole-cell and extracellular proteins for SDS-PAGE: The test strains were cultivated at least duplicated to prepare the synchronous culture. For each synchronous culture, 100 µL was inoculated into 15 mL brain heart infusion broth and incubated at rotated incubator for 24 h (at 37 °C, 150 rpm). Each sample was centrifuged for 5 min at 12.100 rpm and the pellet collected was resuspended in 200 µL of CellLytic™ B-II bacterial cell lysis/extraction reagent (sigma). The suspension was incubated for 30 min at room temperature. Afterwards, the sample was again centrifuged and 80 µL from each sample was transferred into a new 1.5 mL Eppendorf tube. Then, 25 µL of modified SDS-samples buffer (0.06 M tris-HCl, 2.5 % glycerol, 0.5 % SDS, 1.25 % β-mercaptoethanol, 0.1 % TCA, 10 mM urea, 1 mM EDTA) was added and the whole mixture was vortexed to ensure good homogenization. The prepared samples were kept on a boiling water bath for 5 min and denatured proteins stored at -70 °C until the electrophoresis is carried out.

For the extraction of extracellular protein was performed by applying minor modification to the method recommended by Wessel and Flugge[32]. For this processing, the culture supernatants of all *S. aureus* strains were passed through a cellulose acetate membrane filter (*Sartorious*) with a diameter of 0.25 mm and it was stored at -70 °C until the electrophoresis was carried out. A methanol-chloroform precipitation was applied in an Eppendorf tube containing 400 µL of methanol, 200 µL of chloroform and 300 µL of distilled water and the mixture was centrifuged for 5 min

at 10.700 rpm. After centrifuging, the upper phase was removed and 300 µL of methanol was added. The mixture was stirred and was centrifuged again at 10.700 rpm. Then the supernatant was removed and the precipitated proteins were dried at room temperature. The dried protein was stirred after the addition of 25 µL of modified SDS-sample buffer (pH 6.8) to the Eppendorf tube. Afterwards, pellet proteins were denatured by keeping them in boiling water for 5 min.

SDS-PAGE: The whole-cell and extracellular proteins were subjected to SDS-PAGE in gel slabs of 1 mm thickness (3.5 cm, 4 % stacking and 15.5 cm, 12 % resolving gels) as described by Laemmli[33]. Electrophoresis was performed with a discontinuous buffer system in a UVP vertical electrophoresis unit (Cambridge, UK). The gel was run at 30 mA until the bromophenol blue marker had reached the bottom of the gel. Protein molecular masses were calculated on the basis comparison with the following standards (PageRuler™ Protein Ladder SDS-PAGE standards, fermentas, molecular weight range 10-200 kDa). After electrophoresis the gels rinsed out for 20 min in an isopropanol-acetic acid-water (1:3:6) solution, then for 5 min in methanol-acetic acid-water (3:1:6) solution. Then, the gels were stained for 6 h in 0.01 % (w/v) Coomassie Brilliant Blue R-250. Afterwards, the gels were destained in a methanol-acetic acid-water (3:1:6) mixture until protein bands became clearly visible.

Extraction of whole-cell proteins for Native-PAGE: After overnight incubation at 37 °C (150 rpm) in brain heart infusion broth of all *S. aureus* strains, each sample was centrifuged for 5 min (12.100 rpm, at 4 °C) and the pellet washed three time with distilled water. Then the washed pellet was resuspended with a volume of sterile distilled water equivalent to pellet volume. The sample was then placed in a water bath containing melting ice and was subjected to sonication for 5 min from an ultrasonic probe (Jencons Scientific Ltd., UK) emitted 45 W at maximum power. The cell lysates were added to Native-sample buffer (0.06 M tris-HCl, 2.5 % glycerol, 0.5 % 1 mM EDTA, bromophenole blue) at a proportion 2:1 and the native whole-cell proteins were extracted.

Native-PAGE: The extracted native whole-cell proteins were subjected to Native-PAGE in gel slabs of 1 mm thickness (3.5 cm, 4 % stacking and 15.5 cm, 12 % resolving gels) according to Laemmli[33]. Electrophoresis was performed with a discontinuous buffer system in a UVP vertical electrophoresis unit (Cambridge, UK) at 4 °C and subsequently steps were carried out as mentioned above.

Protein profile analysis: The gels were scanned *via* a high resolution scanner (HP 3500 C, Hewlett Packard Co.) and the molecular weight of each band was determined by one-dimensional analysis software (Lab Image Version 2.6, Halle, Germany). Data were coded as 0 (absent) and 1 (present). A hierarchical cluster analysis was performed using the average linkage method and correlation coefficient distance. The dendrogram, based on the whole-cell and extracellular protein patterns of the test strains, was constructed by the program Minitab for Windows, version 14.20 (Minitab Inc. Pennsylvania, USA).

RESULTS AND DISCUSSION

In this investigation, the morphological and biochemical tests indicated that the isolates were the member of *S. aureus* species. Of them, 32 (84.21 %) were coagulase-positive, 23 (60.52 %) were MRSA and 15 (39.47 %) MSSA. The results of the antibiogram revealed that all of the isolates identified as *S. aureus* were susceptible to vancomycin, but they were resistant to β-lactams, quinolones, aminoglycosides, macrolid, chloramphenicol, piperacillin + tazobactam and trimetroprim + sulfamethoxazole. Moreover, plasmid profile analysis results confirmed that the strains had only one plasmid, molecular weight 13.868 bp (data not shown).

The whole-cell and extracellular protein profiles of *S. aureus* strains are given in Figs. 1 and 2, respectively. In the Fig. 1 indicated that the whole-cell protein profiles of each one of the *S. aureus* strains had 10 major protein bands. SDS-PAGE analyses of whole-cell proteins exhibited that there are major similarities between all *S. aureus* strains in their high-molecular-mass range (> 60 kDa). However, the minor distinctive proteins were observed both in the low (< 60 kDa) and high-molecular mass range (between 70-100 kDa). Similarities in the profiles of *S. aureus* strains were manifested by the existence of bands 1, 2, 3, 4, 6, 7, 9. Besides, the strains ND-26 and ND-29 (lanes 11 and 12) were distinguished from the other strains by the presence of two bands marked 5 and 8. The strain NA-10 (lane 6) was discernable from all the strains because of the presence of single band 10. Moreover, the reference strains (lanes 15, 16 and 17) were similar to protein patterns of the strains isolated from different body sides.

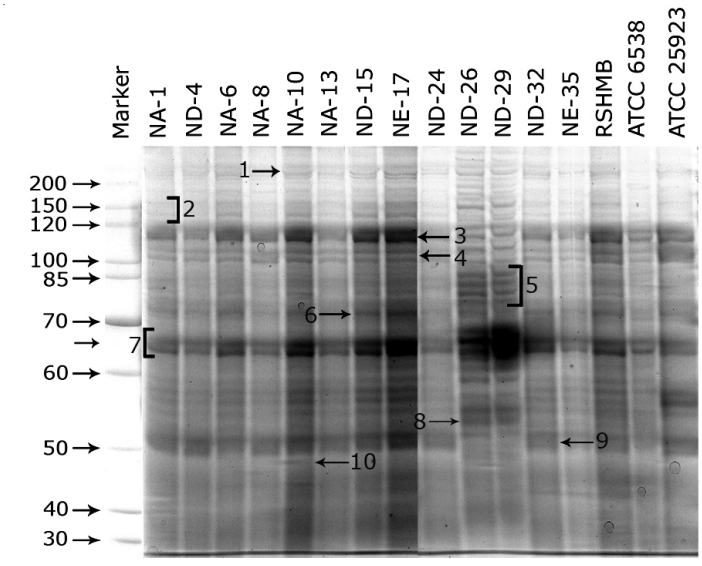

Fig. 1. SDS-PAGE of whole-cell protein profiles of *S. aureus* strains. Line 1; molecular weight marker (10-200 kDa) and lanes 2-17; *S. aureus* strains

Although the protein banding of all strains to be very similar, some minor differences may be observed among strains (Fig. 2). The comparison of extracellular protein patterns revealed that there are major similarities between *S. aureus* strains in their high-molecular-mass range (> 70 kDa). However, some minor differences were observed in the low (< 60 kDa). Such as, the strains consisted of dominant protein bands marked as 11, 13 and 14. The strains NA-13, ND-15 and NE-17 (lanes 7, 8 and 9) were distinguished from the other strains by the presence of binary bands marked as 15. Besides, the strains ND-15 and NE-17 (lanes 8 and 9) had two discriminative bands marked as 17 and 18. The strain ND-10 (lane 3) was discernable from all the strains because of the presence of single band 19.

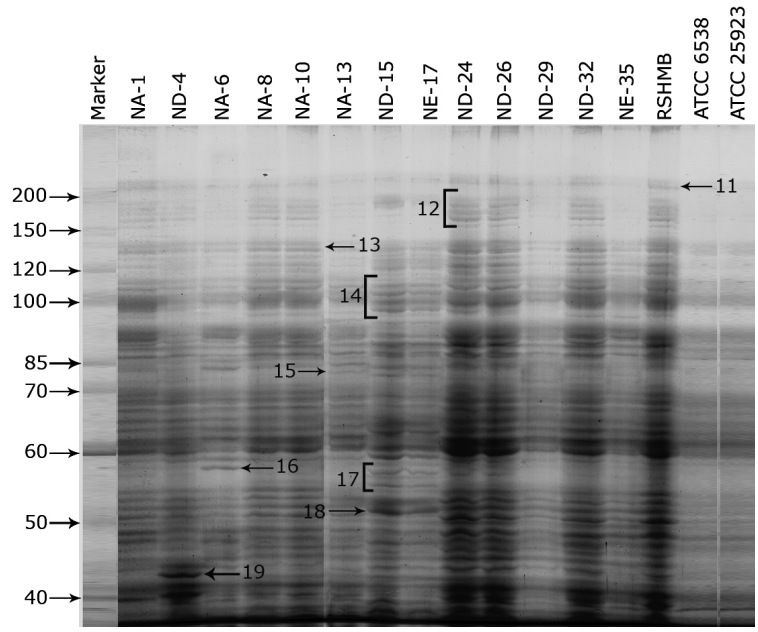

Fig. 2. SDS-PAGE of extracellular protein profiles of *S. aureus* strains. Line 1; molecular weight marker (10-200 kDa) and lanes 2-17; *S. aureus* strains

Native-PAGE of whole-cell protein patterns of *S. aureus* strains is shown in Fig. 3. The results of Native-PAGE analysis showed that several strains had major characteristic band patterns, but there was reliable the discriminative banding for *S. aureus* at the strain levels. For example, the standard *S. aureus* strains (line 14, 15 and 16) had very similar banding. They distinguished from all the strains because of the presence of single band 22. The strain ND-29 (lane 11) was distinguished from the other strains by the presence of twofold protein band signed by 23. The strains ND-26 and ND-29 (lanes 10 and 11) had the distinctive single band numbered as 25 except for other strains. Also, the band signed by 27 for the strain NA-6 (line 3), the band marked as 28 for the strains ND-24, ND-26 and ND-29 (lines 9, 10, 11)

and the band marked as 30 for the strain NA-13 (line 6) and ND-29 (line 11) were discriminative. The strains NA-1, NA-6, ND-32 and NE-35 (lines 1, 3, 12 and 13) had the protein band numbered by 33. Moreover, the strains ND-15 (line 7), NE-17 (line 8) and the reference strains (lanes 14, 15 and 16) were distinguished from other strains by the existence of bands 34 and 35.

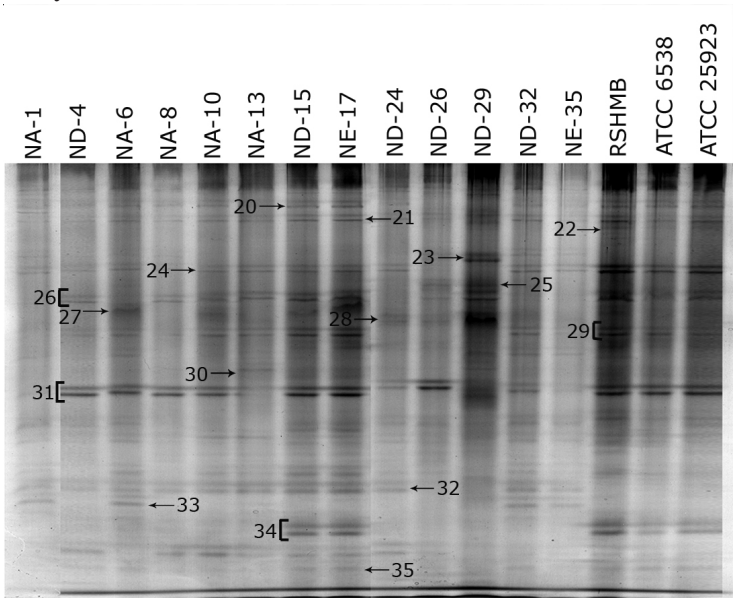

Fig. 3. Native-PAGE of whole-cell protein profiles of *S. aureus* strains. Lane 1-16; *S. aureus* strains

The numerical analysis of SDS-PAGE profiles of whole-cell proteins extracted from the strains of *S. aureus* based on average linkage and correlation coefficient distance yielded a dendrogram, consisting of two clusters (I and II) at similarity levels of 80.79 % or above (Fig. 4). The cluster I included 16 *S. aureus* strains, sharing in the intra-cluster similarity levels of 100 %. The cluster II had only two strains and they displayed at similarity values of 90.10 % or above. The cluster analysis of SDS-PAGE profiles of the extracellular proteins obtained from the strains of *S. aureus* (based on average linkage and correlation coefficient distance) revealed also two clusters (I and II) at similarity levels between 87.49 and 100 % (Fig. 5). The cluster I comprised 8 *S. aureus* strains at similarity degrees 100 %. Also, the cluster II had 8 strains at similarity values changed between 94.26 and 100 %.

Besides, the hierarchical cluster analysis of Native-PAGE profiles of whole-cell proteins obtained from the strains of *S. aureus* using average linkage and correlation coefficient distance generated a dendrogram, containing two groups (I and II) at similarity levels more than 76.76 % (Fig. 6). The group I consisted 3 *S. aureus* strains at similarity levels 84.50 % or above. Subsequently, the group II had 13 strains at similarity values changed between 88.20 and 100 %. The group II divided

into two subgroups (IIa and IIb). Subgroup IIb had 9 strains and they together exhibited the highest similarity of protein profiles. The members of subgroup IIa were similar to each other, sharing many common bands as reflected in the high intra-cluster similarities (Fig. 6).

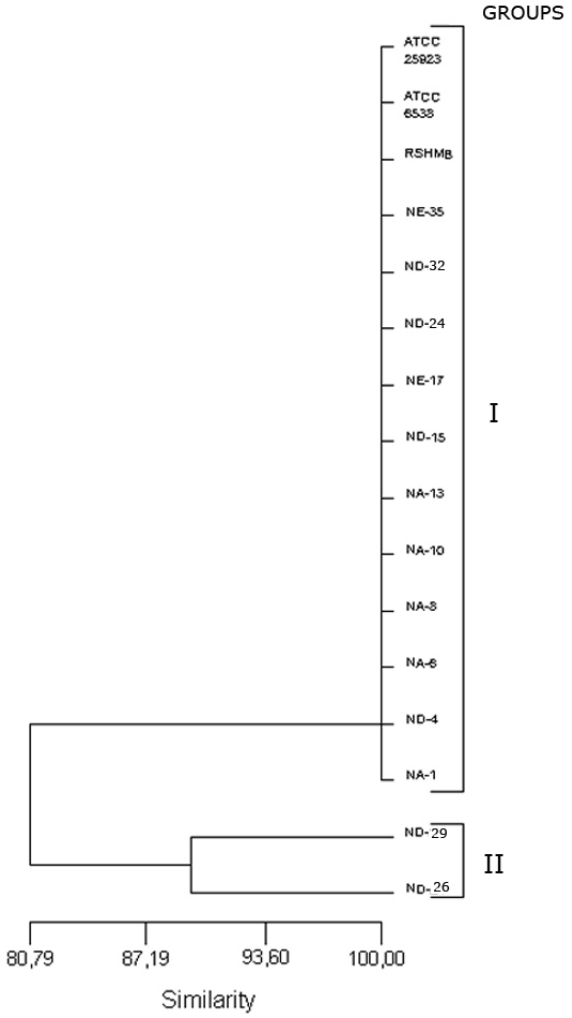

Fig. 4. Grouping of *S. aureus* strains studied using hierarchical cluster analysis (average linkage and correlation coefficient distance) based on whole-cell protein profiles obtained by SDS-PAGE

The results of biotyping and antibiogram indicated that the use of two methods together was insufficient for the discrimination at the strain level. The antibiogram results showed that the isolates were resistant for all the tested antibiotics except for vancomycin and this pattern of antibiotic resistance is an indicator that the isolates might have originated from nosocomial resources. Also, the presence of

only one plasmid in whole strains might be explains multiple antibiotic resistance could be because of the existence of this plasmid, containing a number of antibiotic resistance genes. Furthermore, plasmid profile analysis technique had not sufficient capacity for the differentiation of *S. aureus* strains due to loss or gain of plasmid DNA. In this vein, our results were in agreement with previous studies[13-15,34,35].

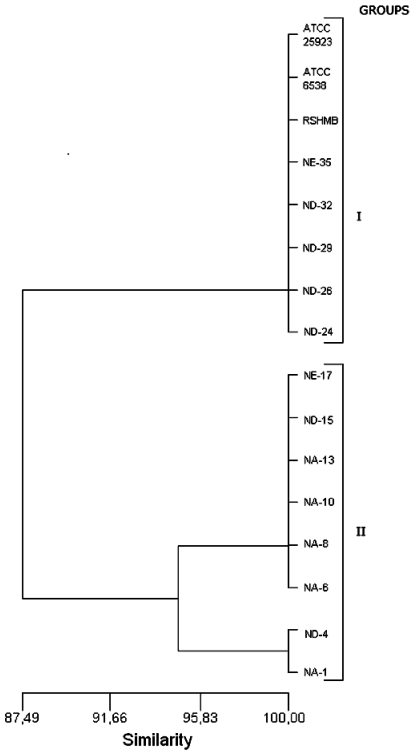

Fig. 5. Grouping of *S. aureus* strains studied using hierarchical cluster analysis (average linkage and correlation coefficient distance) based on extracellular protein profiles obtained by SDS-PAGE

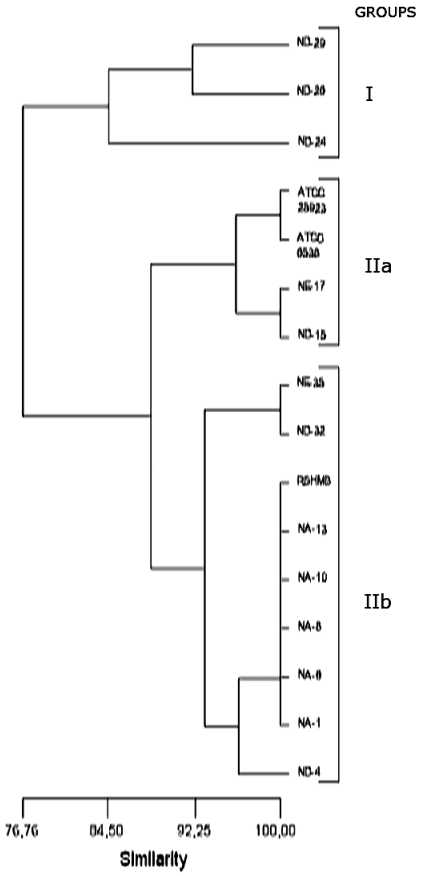

Fig. 6. Grouping of *S. aureus* strains studied using hierarchical cluster analysis (average inkage and correlation coefficient distance) based on whole-cell protein profiles obtained by Native-PAGE

Several types of electrophoretic methods based on whole-cell and extracellular protein profiles have been used for the characterization and the discrimination of *S. aureus* strains and other *Staphylococci* isolated from various sources[19,20,24,25,34,36-38]. The researchers reported that whole-cell and extracellular protein patterns obtained

by SDS-PAGE of *Staphylococcus* species can provide valuable epidemiological information for differentiation at the species level, but the differences were insufficient for the discrimination at the strain level[19,20,39]. Present results were in agreement with the finding of previous studies as cited above. There have been barely reports regarding Native-PAGE typing of *S. aureus* and some bacterial species with whole-cell and extracellular proteins[25,26]. The authors had purposed that Native-PAGE of whole-cell and extracellular protein supplied extra banding patterns of the differentiation at the species level and not below, comparing to SDS-PAGE of whole-cell proteins. Present findings were in agreement with the results of two authors for the discrimination at the species level. However, this study supposed that Native-PAGE methods of whole-cell proteins provide a good degree of differentiation at the strain levels. The difference was due to use of a good protein extraction procedure and optimum standardization conditions of method. Furthermore, Native-PAGE of extracellular protein lysates offer additional characteristic banding patterns for the differentiation of MRSA and other *S. aureus* strains (unpublished data).

Recently, several valuable papers suggested that the application of numerical analysis, coupled with the utilization of a standardized identification system instead of simple quantitative comparison of protein patterns provides an effective approach to the investigation of taxonomic relationships among many microorganism species[14,18,19,21,40]. Here in the present study, MINITAB program was used to analyze the data because of the difficulties in the visual interpretation of the bands obtained in SDS-PAGE of whole-cell and extracellular proteins, as well as in Native-PAGE of whole-cell proteins. The cluster analysis of SDS-PAGE profiles of whole-cell proteins and extracellular of *S. aureus* strains displayed similar pattern showing more than 80.79 and 87.49 %, respectively (Figs. 4 and 5). The numerical analysis results using by SDS-PAGE method of both whole-cell and extracellular protein profiles confirmed that each cluster had slight discriminative protein banding. But, the numerical analysis of Native-PAGE of whole-cell proteins was a good method of distinguishing between MRSA and other ordinary *S. aureus* strains. For example, the cluster I numbered 3 of MRSA strains numbered by ND-24, ND-26 and ND-29 (lines 9, 10 and 11), sharing two distinctive protein bands marked as 23 and 28. However, all members of the cluster II were MSSA, having many common bands as reflected in the high intra-cluster similarities. Present results revealed that the same clone was responsible for most cases of MRSA and ordinary *S. aureus* epidemic among Van (Turkey) hospitals even though the strains collected from different hospitals. The finding was in coherent with the results of the other genotyping assays[16,25].

The use of the molecular techniques as a typing method, including PCR-based techniques, ribotyping, DNA sequencing and DNA-DNA hybridization, for discrimination of MRSA and ordinary *S. aureus* strains needs to be exactly standardization and the methods not performed traditional laboratory condition. Therefore, these methods require expert personal who have knowledge of the standardization and interpretation. Besides, some of them are dangerous due to the use of hazardous

chemicals and radioactive method of labeling and they are fairly laborious and very expensive. On the other hands, SDS-PAGE and Native-PAGE techniques can be made less hazardous because of not using the poisonous substances and they are relatively inexpensive. In this sense, the protein fingerprinting techniques may be used as an alternative to establishing PCR-based methods.

In conclusion, the study suggested that together application at least two different polyacrylamide gel electrophoresis (PAGE) techniques can be offer accurate and an effective approach to the investigation of taxonomic relationships within human pathogenic *S. aureus* strains. Afterwards, the results reinforced that Native-PAGE of whole-cell proteins may be used as a confirmative method for differentiation among MRSA and other *S. aureus* isolates.

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