Turk J Biol
24 (2000) 817–823

Availibility of use of Total Extracellular Proteins in SDS-PAGE for Characterization of Gram-Positive Cocci

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Received: 11.02.1999

Abstract: There are no reports in the literature dealing with gram-positive cocci typing by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of extracellular proteins. In this study, *Staphylococcus aureus, Staphylococcus warneri, Staphylococcus chromogenes, Micrococcus lysodeikticus* and Micrococcus ssp. of the Micrococcaceae family, and *Pediococcus halophilus, Streptococcus* ssp. and *Enterococcus faecalis* of the Streptococcaceae family were characterized by SDS-PAGE of whole cell proteins (WCPs) and total extracellular proteins (TEPs). Comparison of SDS-PAGE of WCP profiles with banding patterns produced by that of TEPs provided additional criteria and characteristic banding patterns for the study of the characterization of gram-positive cocci. This method was found to be easy to use and interpret and provided a rapid, reproducible and discriminative method for typing gram-positive cocci.

Key Words: SDS-PAGE, gram-positive cocci, characterization.

SDS-PAGE'de Hücre Dışı Proteinlerinin Gram-Pozitif Kokların Karakterizasyonu İçin Kullanılması

Özet: Sodyum dodesil sülfat-poliakrilamid jel elektroforez (SDS-PAGE)'de hücre dışı proteinler (TEP) kullanılarak gram-pozitif kokların tiplendirimi ile ilgili herhangi bir rapora şu ana kadar rastlanmamıştır. Bu çalışmada; Micrococcaceae familyasına ait *Staphylococcus aureus, Staphylococcus warneri, Staphylococcus chromogenes, Micrococcus lysodeikticus* ve Micrococcus ssp., ve Streptococcaceae familyasına ait *Pediococcus halophilus, Streptococcus* ssp. ve *Enterococcus faecalis* SDS-PAGE'de hem hücre proteinleri hem de hücre dışı proteinler kullanılarak karakterize edilmiştir. Hücre proteinleri kullanılarak yapılan SDS-PAGE'de elde edilen protein bantları hücre dışı proteinler kullanılarak yapılan SDS-PAGE ile karşılaştırıldığında hücre dışı proteinler kullanılarak yapılan SDS-PAGE'in gram-pozitif kokların karakterizasyonu yönünde daha fazla ayırım gücüne sahip olduğu gösterilmiştir. Uygulanması ve protein bandlarının yorumlanması oldukça kolay olan bu metod gram pozitif kokların tiplendirilmesi amacıyla hızlı ve ayırım gücü fazla olan bir yöntem olarak önerilmektedir.

Anahtar Sözcükler: SDS-PAGE, gram-pozitif kok, karakterizasyon.

Introduction

The most widely accepted routine test for identifying staphylococci is based on their ability to produce acid from glucose under anaerobic conditions, distinguishing them from micrococci, which lack this ability (2, 15, 16). The separation of micrococci and staphylococci by the classical oxidation-fermentation test, however, raises several problems, such as the inability of certain species of staphylococci to produce acid from glucose under anaerobic conditions or produce only small amounts of acid. There are also some micrococci that anaerobically produce small to moderate amounts of acid from glucose (7, 9, 13). In addition methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcus (VRE), which are nosocomial pathogens, have been important subjects in recent years. For this reason, the typing of MRSA and other gram positive cocci is assuming increasing importance. In recent years, MRSAs have been the most significant cause of hospital-based infections and have been a cause for concern in most hospitals (1, 3, 4). In addition, coagulase negative staphylococcus (CNS), which was not considered until recent years, has become one of the most important subjects since it was found to be one of the nasocomial pathogens. Although the pathogenic role of coagulase-negative staphylococci is now well established, the clinical significance of the various species is still being defined. We should not disregard any of these organisms until their clinical significance is determined, and since they are frequently opportunistic pathogens, we may never completely resolve this question of significance. Other members of the family Micrococcaceae are also being identified as oppurtunistic pathogens (8). Conventional methods used to identify species of staphylococci involve many tests that require the preparation of a variety of special media and reagents and 3 to 5 days of incubation to obtain results. A further complication is the range of reactivity within each species with many of the test systems. There are different commercial test kits for the identification of CNS at species level and the reliability of these phenotype-based systems varies between 50 and 70% discrimination (6). The analysis of whole cell profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has recently been established as a useful method for the identification of Staphyococcal and *Bacillus* species (4, 5, 12). To date, SDS-PAGE of whole-cell proteins has been used for typing staphylococci but there have been no studies report issued yet regarding the typing of gram-positive cocci at genus level or the typing by SDS-PAGE of extracellular proteins.

In this study, SDS-PAGE of both whole cell proteins (WCPs) and total extracellular proteins (TEPs), which allow quicker differentiation without the expense or specialist expertise required by more sophisticated methods, were used for gram positive cocci typing.

Materials and Methods

Bacterial Strains and Media

All cultures were grown at 35°C for 24 hr in BHI (Brain Heart Infusion, Difco) agar and propagated at least twice before use. The reference bacteria which were used in our research were kindly provided by the following researchers: *Staphylococcus aureus* ATCC 25923, *Staphylococcus warneri* ATCC 27836, and *Staphylococcus chromogenes* ATCC 43764 from Dr

Jeffrey L. Watts (Lousiana State University, Agricultural Center, Homer, LA, USA); *Micrococcus lysodeikticus* from Dr. Jungi Sakurada (Jikei University, School of Medicine, Department of Bacteriology, Tokyo, Japan); *Micrococcus* spp. NCTC 1630 from Refik Saydam Hıfzısıhha Institute, Ankara, Turkey); *Pediococcus halophilus* isolated by the authors from Turkish kasher cheese; clinical isolates of *Streptococcus* spp and *Enterococcus faecalis* ATCC 29212 from Dr. Ahmet Başustaoğlu (Gülhane Military Medical Academy, Infectious Disease and Clinical Microbiology Department, Ankara, Turkey).

Preparation of Whole Cell Proteins (WCPs)

For each culture, a loopful of overnight growth from a BHI agar plate was suspended in 15 ml BHI broth and incubated in a rotating incubator for 7 hr (at 35°C, 150 rpm). The samples were then transferred into eppendorf tubes and centrifuged for 3 minutes at 12,100 rpm. The collected cells were washed three times with sterile distilled water. The washed cells were stirred after the addition of 25µl SDS sample buffer (0.06 M Tris, 2.5% Glycerol, 0.5% SDS, 1.25% β-mercaptoethanol and 0.001% bromophenol blue) and the proteins were denatured in boiling water for 5 minutes. The supernatant was then centrifuged again for 3 minutes at 12,100 rpm, collected in an eppendorf tube and kept at -50°C until electrophoresis was carried out.

Preparation of extracellular proteins (TEPs)

The culture supernatants were passed through a cellulose acetate membrane filter (Sartorius) with a diameter of 0.2µm ad stored at -50°C until electrophoresis was carried out. Methanol-chloroform precipitation was performed, applying modifications to the method recommended by Wessel and Flugge (18). In brief, the volume of the sample increased; after the addition of 400µl methanol, 200µl chloroform and 300µl distilled water, it was shaken and centrifuged for three minutes at 10,700 rpm. After centrifugation, before it went into the intermediate phase, the supernatant was removed carefully, and after adding 300µl methanol and stirring, it was centrifuged again at 10,700 rpm. After the supernatant was removed, the precipitated proteins were dried by an air current and stirred after the addition of 25µl SDS-sample buffer. Afterwards, the proteins were denatured in bailing water for 5 minutes.

SDS-PAGE

The denatured proteins were analyzed by SDS-PAGE. This method used a 2 cm layer of 4% acrylamide stacking gel and a 10 cm layer of 10% arcylamide separating gel. Sigma wide-range marker was used as the molecular weigh standard in SDS-PAGE. Electrophoresis was performed with a discontinuous buffer system in a BRL gel apparatus model V16-2BRL, Gaithersburg MD, USA. The gel was run at a constant current of 35mA until the bromophenol blue marker reached the bottom. The gels were then stained with Coomassie Brilliant Blue (Sigma) (11).

Results and Discussion

In adition to traditional biochemical tests, in this study, we evaluated the suitability for use of total extracellular proteins (TEPs) and whole-cell proteins (WCPs) in SDS-PAGE as a routine typing system for gram-positive cocci. The development of new methods and the modification

of old ones are desirable since existing methods often give ambiguous or misleading results. Analysis of different gram positive cocci by SDS-PAGE gave reproducible whole-cell (Figure 1) and extracellular polypepytide profiles (Figure 2). As far as the band profiles of WCPs in SDS-PAGE were concerned, common characteristic bands of 6.7 kD were observed in all gram-positive cocci except *Micrococcus lysodeikticus* (as a result of correspondence with Dr. Sakurada, the possibility of changes in the genetic stability of this microorganism has been stated). In addition, different gram-positive bacteria were distinguished by the following observations: 21, 23 and 25 kD characteristic protein bands in different strains of staphylococci in lines 1, 2 and 3, respectively; a 203 kD dominant band in *Micrococcus* spp. in line 5; 13 and 14 kD twin bands as well as a 203 kD band of *Pediococcus halophilus* in line 6; and 18 and 35 kD characteristics bands of *Streptococcus* spp. and *Enterococcus faecalis* in lines 7 and 8 respectively. We modified this system in order to obtain better discrimination between different gram-positive cocci with the formation of characteristic band patterns specific to each genus, which may be the result of the high level expression of proteins. It seems clear that our modified typing system in SDS-PAGE by use of extracellular proteins gave much more characteristic dominant bands than SDS-PAGE of whole-cell protein profiles and this system can be used as an effective typing method.

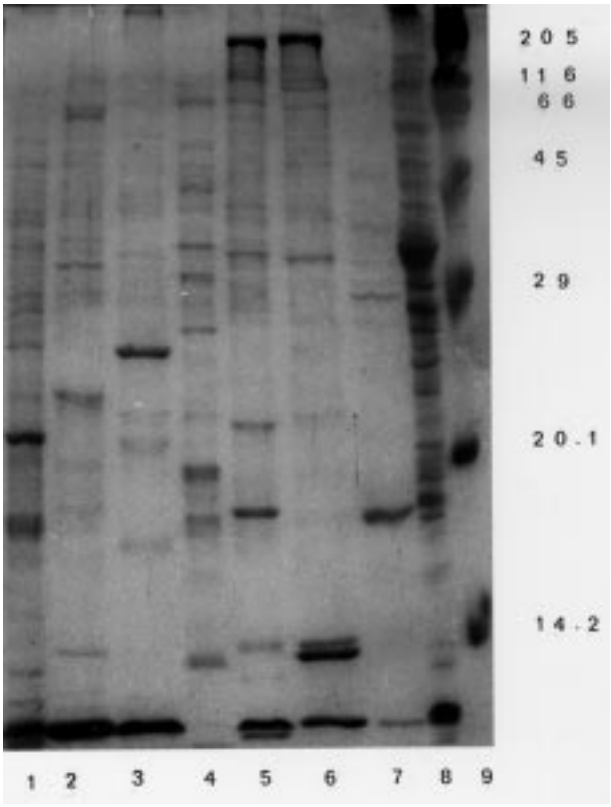

Figure 1. SDS-PAGE of whole cell proteins of gram-positive cocci. Line 1: *Staphylococcus aureus* ATCC 25923, 2: *Staphylococcus warneri* ATCC 27836, 3: *Saphylococcus chromogenes* ATCC 43764, 4: *Micrococcus lysodeikticus*, 5: Micrococcus ssp. NCTC 1630, 6: *Pediococcus halophilus*, 7: *Streptococcus* ssp., 8: *Enterococcus faecalis* ATCC 29212, 9: Molecular weight marker of SDS-PAGE (Sigma wide-range marker).

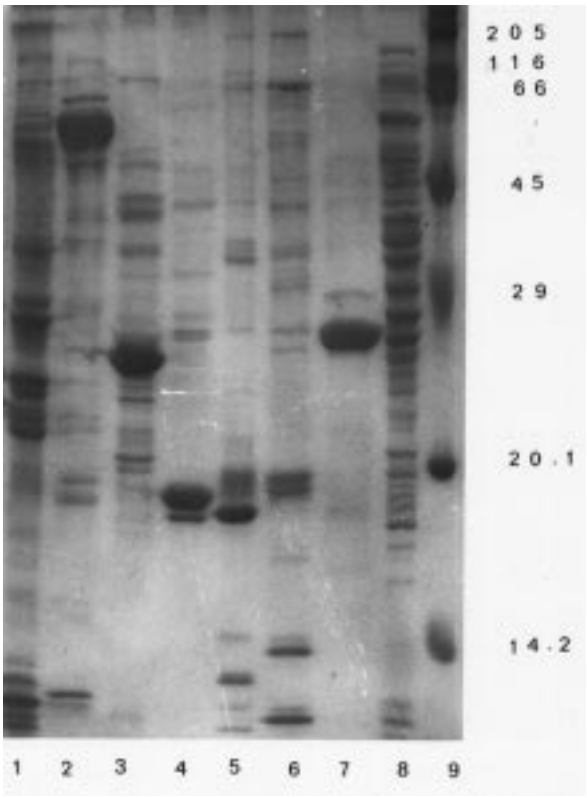

Figure 2. SDS-PAGE of whole cell proteins of gram-positive cocci. Line 1: *Staphylococcus aureus* ATCC 25923, 2: *Staphylococcus warneri* ATCC 27836, 3: *Saphylococcus chromogenes* ATCC 43764, 4: *Micrococcus lysodeikticus*, 5: Micrococcus ssp. NCTC 1630, 6: *Pediococcus halophilus*, 7: *Streptococcus* ssp., 8: *Enterococcus faecalis* ATCC 29212, 9: Molecular weight marker of SDS-PAGE (Sigma wide-range marker).

This is illustrated by the 208 kD protein band of *Staphylococcus aureus* ATCC 25923 in line 1, which is not seen in other strains; 55 and 26 kD highly expressed protein bands of *Staphylococcus warneri* ATCC 27836 and *Staphylococcus chromogenes* ATCC 43764 in lines 2 and 3, respectively; 18 kD, 17 kD and 28 kD bands of *Micrococcus lysodeikticus, Micrococcus* spp. NCTC 1630 and *Streptococcus* spp. in lines 4, 5, and 7 respectively; and highly expressed 6.7 kD and 13 kD bands of *Pediococcus halophilus* in line 6. These data demonstrate the suitability of the typing scheme because of the presence of the highly expressed protein bands.

Our results and those of previous studies (4, 10, 14) clearly show that electrophoretic methods can provide valuable epidemiological information that may be used in isolation. As a result, we can conclude that the bands obtained in SDS-PAGE of whole-cell proteins can readily provide data for the establishment of typing schemes (4, 6, 17), but these are less discriminatory than SDS-PAGE of extracellular proteins. Nevertheless, the patterns obtained by TEP profiles were much easier to interpret because of the presence of characteristic protein bands that could be used for differentiation. These profiles provide a rapid procedure for the characterization of gram-positive cocci because only one hour is required for extraction of a 7 hr rotated culture, and this is a great advantage when compared to other typing methods. We

also evaluated the suitability of SDS-PAGE of TEPs as a typing scheme in distinguishing 17 *Staphylococcus aureus* references at strain level and we have shown the availability of the use of total extracellular proteins in SDS-PAGE (unpublished data).

Acknowledgement

We gratefully acknowledge İsmet Berber for his excellent technical assistance.

References

1. Archer, G.L., and C.G. Mayhall. Comparis on of epidemiological markers used in the investigation of an outbreak of methicillin-resistant *Staphylococcus aureius* infections. J. Clin. Microbiol. 18: 395-399, 1993.
2. Baird-Parker, A.C. A classification of micrococci and staphylococci based on physiological and biochemical tests. J. Gen. Microbiol. 30: 409-427, 1963.
3. Birnbaum, D., Kelly, M., and A.W. Chow. Epidemiological typing systems for coagulase negative staphylococci. Infect. Cotrol. Hosp. Epidemiol. 12: 319-326, 1991.
4. Clink, J., and T.H. Pennington. Staphylococcal whole-cell polypeptide analysis: evaluation as a taxonomic and typing tool. J. Med. Microbiol. 23: 41-44, 1987.
5. Çökmüş, C., and A.A. Yousten. Characterization of *Bacillus sphericus* strains by SDS-PAGE. J. Invest. Pathol. 64: 267-268, 1994.
6. Dryden, M.S., Talsani, H.G., Martin, S., Cunningham, M., Richardson, J.F., Cooksan, B., Marples, R.R., and I. Philips. Evaluation of methods for typing coagulase negative staphylococci. J. Med. Microbiol. 37: 109-117, 1982.
7. Evans, J.B., and W.E. Kloos. Use of shake cultures in a semisolid thioglycolate medium for differentiating staphylococci from micrococci. Appl. Microbiol. 23: 326-331, 1972.
8. Fosse, T., Peloux, Y., Granthil, C., Toga, B., Bertrando, J., and M. Sethian. Meningitis due to *Micrococcus luteus*. Eur. J. Clin. Study Treat. Infect. 13: 280-281, 1986.
9. Kloos, W.E., and K.H. Schleifer. Isolation and characterization of Staphyloccocci from human skin. II. Description of four new species: *Staphylococcus werneri, Staphyococcus capitis, Staphylococcus haminis* and *Staphylococcus simulans*. Int. J. Syst. Bacteriol. 25: 62-79, 1975.
10. Krikler, S.J., Pennington, T.H., and D. Pertie. Typing of strains of *Staphylococcus aureus* by western blot analysis of culture supernatants. J. Med. Microbiol. 21: 169-171, 1986.
11. Laemmli, U.K. Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature (London), 227: 680-685, 1970.
12. Pennington, T.H., Harker, C., and F. Thomson-Carter. Identification of coagulase negative staphylococci by using sodium dodecyl sulfate polyacrylamide gel electrophoresis and rRNA restriction patterns. J. Clin. Microbiol. 29: 390-392, 1991.

13. Schleifer, K.H., and W.E. Kloos. Isolation and characterization of stamphylococci from human skin. I Amended description of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*, and description of three new species: *Staphylococcus cohnii*, *Staphylococcus haemolyticus* and *Staphylococcus xylosus*. Int. J. Syst. Bacteriol. 25: 50-61, 1975.

14. Stephenson, J.R., Croak, S.J., and S. Tobaqchali. New method for typing *Staphylococcus aureus* resistant to methicillin based on sulphur-35 methionine labelled proteins, its application in an outbreak. Brit. Med. J. 293: 581-588, 1986.

15. Subcommittee on taxonomy of staphylococci and micrococci. Recommendations. Int. J. Syst. Bacteriol. 15: 109-110, 1965.

16. Subcomittee on taxonomy of staphylococci and micrococci. Minutes of meeting. Int. J. Syst. Bacteriol. 15: 109-110, 1971.

17. Thomson-carter, F.M., and T.H. Pennington. Characterization of methicillin-resistant isolates of *Staphylococcus aureus* by analysis of whole cell and exported proteins. J. Med. Microbiol. 28: 25-32, 1989.

18. Wessel, D., and U.I. Flugge. A method for the quantitative recovery of protein in dilute solution in presence of detergents and lipids. Anal. Biochem. 138: 141-143, 1984.