RESEARCH LETTER

Combination of capillary electrophoresis, PCR and physiological assays in differentiation of clinical strains of Staphylococcus aureus

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Abstract

Fast, sensitive and cheap determination of pathogenic bacteria is extremely important in many branches, for example biotechnology, quality control, analysis of samples and antimicrobial therapy. The development and application of analytical techniques in practice could provide new possibilities in this regard. The bacterial pathogen Staphylococcus aureus is responsible for a significant amount of human morbidity and mortality. Rapid and sensitive determination is therefore very important. In the present study, novel methods, based on capillary zone electrophoresis and (as confirmation of these results) molecular analysis of a part of the coag gene, were developed for identification and differentiation of three S. aureus strains. The electrophoretic measurements rely on the differential mobility of bacteria in the fused silica capillary under the direct current electric field. To perform coagulase gene typing, the repeated units encoding hypervariable regions of the S. aureus gene were amplified using the PCR technique followed by restriction enzyme digestion and analysis of restriction fragment length polymorphism patterns as well as sequencing. Finally, the results of electrophoretic measurements with molecular analysis were compared.

Introduction

Staphylococcus aureus is perhaps the greatest nosocomial pathogen of our time. This Gram-positive bacterium causes a wide range of human diseases ranging from minor skin infections, such as folliculitis and impetigo, to more invasive diseases including endocarditis, sepsis, and toxic shock syndrome (Weems, 2001). Staphylococcus aureus is the leading cause of infectious complications within the hospital environment and the incidence of multidrug resistant isolates is increasing (Reniere et al., 2007). An increasing number of infections are related to medical developments, including the use of joint prostheses, immunosuppressants and catheters (Casey et al., 2007). These facts warrant the rapid identification of infected and colonized patients as well as the interruption of strain transmission. Control of S. aureus infection can be accompanied by epidemiological typing of S. aureus. This may clarify whether environmental strains from staff members are related to those that cause infection and whether the isolates from one patient belong to one genotype.

Most screening is still carried out using plate-based methods, but because a growing number of strains are resistant to multiple antibacterial chemotherapies, their resistance phenotypes are only of little discriminatory value. Therefore, alternative methods such as broth culture, chromogenic media, rapid screening kits, molecular assays and automated systems are increasingly being used (Casey et al., 2007). Molecular methods are valuable not only in the identification of bacterial strains but also in demonstrating the evolutionary and clonal relationships between the isolates. In the present study we evaluated the capacity of coagulase gene typing to discriminate between unrelated strains of S. aureus. Coagulase production is the principal criterion used in clinical microbiology laboratories for the identification of S. aureus isolates from human infections.
Numerous allelic forms of *S. aureus* coagulase exist, with each isolate producing one or more of these enemy variants (Henderson & Brodie, 1963; Reeves et al., 1981; Jeljaszewicz et al., 1983; Mandolo, 1990). Analysis of the DNA sequences of coagulase genes revealed that the variable sequences exist within the 3’ coding regions of the allelic genes (Kaida et al., 1987, 1989; Phonimdaeng et al., 1999; Armstrong & Schneiderheinze, 2000; Schneiderheinze, 2003; Armstrong et al., 2004; Kłodzińska et al., 2005, 2007) and different *S. aureus* isolates could be differentiated by virtue of these unique nucleotide sequences (Goh et al., 1992). This is a sensitive and specific method for the detection of colonization by *S. aureus*, but the complex nature of the biological samples as well as the low concentrations of analytes demand that cultures may still be needed to confirm positive results. Capillary zone electrophoresis (CZE) is a powerful and versatile separation technique that competes with high performance liquid chromatography when applied to the separation of both charged and neutral species. CZE is characterized by high efficiency, repeatability of results, sensitivity toward substances detected and reliability. This technique is also extremely cost-saving as it requires only small amounts of chemical substrates to be used (Baker, 1995).

In recent years, electromigration techniques and, in particular, capillary electrophoresis (CE), have shown an increasing potential in determination of charged particles such as: erythrocytes, haemoglobin and its variants, serum proteins, urine proteins, lipoproteins, molecular diagnosis and microorganisms (Hjerten et al., 1987; Pfetsch & Welsch, 1997; Okun et al., 1999; Armstrong & Schneiderheinze, 2000; Schneiderheinze et al., 2000; Armstrong & He, 2001; Armstrong et al., 2001, 2004; Shintani et al., 2002; Buszewski et al., 2003; Desai & Armstrong, 2003; Petersen et al., 2003).

Bacterial cell walls and membranes contain numerous proteins, lipid molecules, teichoic acids and lipopolysaccharides, which give them a characteristic charge. In physiological values, i.e. between pH 5 and 7, most bacterial strains are negatively charged, because the number of carboxyl and phosphate groups exceeds the number of amino groups. Therefore, bacterial cells undergo electrophoresis in a free solution with their own mobility depending on the ionic strength and pH of the buffer solution (Kłodzińska et al., 2007).

Presently, two research groups (the groups of Armstrong and Buszewski) (Armstrong & Schneiderheinze, 2000; Schneiderheinze et al., 2000; Armstrong & He, 2001; Armstrong et al., 2001; Buszewski et al., 2003; Desai & Armstrong, 2003; Armstrong et al., 2004; Kłodzińska et al., 2005, 2006a, b, 2007; Szumski et al., 2005, 2007) are working on CZE methods that make simultaneous identification, separation and characterization of microorganisms possible. Armstrong and colleagues (Armstrong & Schneiderheinze, 2000; Schneiderheinze et al., 2000; Armstrong & He, 2001; Armstrong et al., 2001, 2004; Desai & Armstrong, 2003) published a series of papers concerning the mechanistic aspects of the separation process, separation of bacterial aggregates, online monitoring of bacterial migration and other applications. The applications included identification of the causative pathogens of urinary tract infections, the determination of bacterial viability, the quantification of bacteria and the determination of living bacterial cells in consumer products. Buszewski and colleagues (Buszewski et al., 2003; Kłodzińska et al., 2005, 2006a, b, 2007; Szumski et al., 2005, 2007) applied chemical modification of the capillary surface with different monomers, e.g. divinylbenzene, trimethylchlorosilane, acrylamide, or using the monolithic columns for the identification of bacteria. Such an approach resulted in suppressed electroosmotic flow (EOF) and considerably reduced the adsorption of bacterial cells to the capillary wall. Using chemical modification of the capillary surface, Buszewski et al. separated five species of bacteria in 8 min at a *L*<sub>ef</sub> = 8.5 cm distance (Szumski et al., 2005).

Here we have coupled the CE and molecular methods based on the analysis of the coagulase genes for the determination of different strains of *S. aureus*. It is the first time that the electrophoretic behaviour of bacterial cells has been compared with molecular assays. Fast diagnosis of *S. aureus* bacterial strains as well as other microbial-based diseases (i.e. *Helicobacter pylori*) without isolation of the pure cultures is obviously advantageous. Confirmation of the sensitivity of the CZE technique in determining pathogenic bacteria and their future application could be extremely useful for medicine as well as in many different branches of life.

**Materials and methods**

**Bacterial strains**

*Staphylococcus aureus* strains, marked as Sa1, Sa2 and Sa16, were isolated from three different patients from tracheostomy hole, wound and ulceration, respectively. The strains were isolated in the same hospital at the same time.

**Electrophoretic measurements**

**Equipment**

The analyses were performed using the HP<sup>3</sup>DCE system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector. Fused silica capillaries were purchased from Composite Metal Services (Worcester, UK). The spectrophotometric measurements were performed using a Helios spectrophotometer (Unicam, Cambridge, UK) equipped with a quartz cell of 1 cm path length. All the experiments were performed using dynamically modified fused silica capillaries with an internal diameter 75 μm, total.
length \( L_{\text{tot}} = 33.5 \, \text{cm} \) and effective length \( L_{\text{eff}} = 25.0 \, \text{cm} \). The bacterial suspension was directly injected into the CE system.

**Chemicals**

The following chemicals have been used for analyses of bacteria: disodium EDTA, boric acid (analytical grade) (POCh, Gliwice, Poland), Tris (99.5%) and \([2-(\text{N-Morpholino})\text{ethanesulfonic acid}]\) (95%) (Merck, Darmstadt, Germany), and polyethylene oxide (PEO; \( M_w = 600,000 \)) (Aldrich). Deionized water was produced in our laboratory using a Milli-Q water purification system (Milipore, Bedford, MA).

**Microbiological tests**

Bacterial strains were characterized for their morphological/cultural/physiological/biochemical properties: Gram stain and cell morphology; alternative tests for Gram reaction (formation of slime with 3% KOH); and cytochrome oxidase and catalase tests. The coagulase was tested with fresh rabbit plasma [an overnight colony suspended in 0.5 mL of plasma, diluted (1:10) and incubated at 37 °C for 2 h].

**BIOLOG identification and physiological differentiation**

Bacterial strains were differentiated and identified by studying the utilization of different carbon sources contained in the BIOLOG ver. 4.2 test microplates for Gram-positive bacteria (Biolog Inc., Heyward, CA).

**Molecular identification and coagulase gene polymorphism**

The 3'-end region of the coagulase gene was amplified using the nested-primer technique (Goh et al., 1992). The outer primers were COAG1 (5'-ATACTCAACCGACGACCAG-3') and COAG4 (5'-GATTTTGGATGAAGCGGATT-3'), which hybridize to the coagulase gene at sites 1362–1381 and 2859–2878, respectively. The inner primers were COAG2 (5'-CGAGACCAAGATTCAACAAG-3') and COAG3 (5'-AAAGAAACACTCAGTCA-3'), which hybridize to sites 1632–1651 and 2589–2608, respectively. The PCR amplification products were purified using the QIAquick protocol (Quiagen).

The nested PCR product was digested with the restriction endonucleases Alul, EcoRI, HaeIII and TaqI (Invitrogen) according to the manufacturer's recommended protocol. Both the PCR products and the restriction fragment length polymorphism (RFLP) patterns were detected by electrophoresis in 3% agarose gel.

Direct sequencing of PCR products was performed using the primers COAG-2 and COAG-3 as sequencing primers. Sequences were manually aligned with the aid of the SEQUENCHER TW Version 4.1 system (Gene Codes, Ann Arbor, MI). Cycle sequencing was conducted using the ABI PRISM Dye-Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) followed by electrophoresis on an automated sequencer (ABI 373A Stretch, Applied Biosystems). The BLAST database (Altschul et al., 1997) of the National Centre for Biotechnology Information (NCBI) was used to compare the resolved sequences of coagulase gene repeat region of three isolates with known 16S rRNA gene sequences of bacterial strains collected in the GenBank. DNA sequences determined for this study were submitted to the GenBank and accession numbers were given (Table 2).

**Results**

**Application of CZE in the differentiation of different strains of \( S. \) aureus**

The main problems are in electrophoretic analysis of bacteria are separation performance and selectivity of the methods (Buszewski et al., 2003; Kłodzińska et al., 2005, 2006a, b, 2007; Szumski et al., 2005, 2007). Microorganisms can change their surface properties based on interactions with each other and the surrounding environment. This change in surface characteristics dictated how the bacterial cells may interact when forming biofilms or aggregates. By contrast, the fused silica capillary surface shows numerous types of interactions with the solvent and the solutes. In the case of bacteria, strong adhesion and aggregation effects are observed. When the bacteria are migrating in clusters or aggregates, the magnitude of EOF becomes unpredictable leading to a poor repeatability of the migration times. This is common in the case of biomolecules which have hydrophobic, electrostatic, hydrogen bonding and Van der Waals interactions with the capillary wall, giving a strong tendency to adhesion and to poorer resolution.

For three clinical strains of \( S. \) aureus, different migration times and spectra were reported, but no separation was observed for the mixture of the same bacterial strains (Fig. 1). This was probably indicative of reciprocal interactions between individual strains in the mixture. The causes of these interactions are not completely understood but they result in low separation efficiency. Any loss of efficiency is undesirable as it may cause different bacterial cells to migrate together leading to poor resolution and difficulty in quantifying the bacteria.

There are many different approaches which can be undertaken to reduce interactions of microorganisms with the capillary wall (Szumski et al., 2005; Kłodzińska et al.,...
2006a, b, 2007). The simplest approach involves dynamic modification of the internal capillary surface. In our study we added a small amount of PEO to the running buffer. The additives to the electrolyte interact with the internal capillary surface and alter the charge and hydrophobicity. This modification is much easier to use and to optimize than covalent coatings as it is simply prepared by dissolving the appropriate polymer in the buffer solution. Using this method we previously obtained very good separation of different species of bacteria in the biological matrix.

Various buffer solutions were used in the experiments. Thus far, only single and universal solutions capable for analysing a blind sample and identifying microorganisms present in the sample have not been found. The PEO addition to the buffer solution was used to eliminate the unfavourable phenomenon of cell adsorption to the internal capillary surfaces and to suppress the velocity of EOF, which would prevent microbial separation. Due to bacterial cell aggregation, samples were agitated or subjected to ultrasound before performing the injection into the capillary.

We tried to resolve three different clinical strains of S. aureus in a single run. In the case of these bacteria, we took into consideration the strong tendency to self-aggregation and adhesion to many surfaces as a result of their natural behaviour in the environment. Strong bacterial signals were obtained when pure cultures of each of the bacterial strains were injected separately to the CE system (Fig. 1b, Table 1).

A satisfactory separation for two of strains S. aureus was achieved with the buffer solution containing 4.5 mM Tris, 4.5 mM boric acid and 0.1 mM EDTA [Tris–Borate–EDTA (TBE) buffer] in deionized water at pH = 8.53, with an addition of 0.0125% PEO (Mw = 600 000) (Fig. 1c).

On the basis of electrophoretic parameters arising from the charge localized on bacterial cell surface, it is evident that the examined strains S. aureus 1 and S. aureus 16 are almost identical. The strain S. aureus 2 differs considerably in electrical properties, that is why good separation between S. aureus 1 and S. aureus 2 was achieved (Fig. 1c). The peak shape for S. aureus 2 was also highly characteristic. These experiments were compared with data from molecular and physiological assays.

**Table 1.** Migration times and electrophoretic mobilities for the examined bacterial cells (n = 4)

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Migration times t_m (min)</th>
<th>Electrophoretic mobilities (cm² V⁻¹ s⁻¹)10⁻⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus (1)</td>
<td>2.79</td>
<td>−6.90</td>
</tr>
<tr>
<td>S. aureus (16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus (2)</td>
<td>9.03</td>
<td>−0.195</td>
</tr>
</tbody>
</table>

**Molecular and physiological identification and differentiation of S. aureus strains**

The amplification and the sequencing of the 16S rRNA gene of the three investigated bacterial strains allowed to identify these strains and revealed 100% identity to the reference strain S. aureus ATCC 14458 (Accession number DQ997837; GenBank, NCBI) (data not shown). However, additional physiological analysis – test for the resistance to antibiotics (data not shown) and the correspondence analysis (CA) of different carbon sources (BIOLOG) (Fig. 2) – revealed different properties of the investigated strains. CA comparing use of 95 different carbon sources – carbohydrates (Fig. 2a) as well as other carbon sources (organic...
acids, alcohols, amino acids, nucleosides, amides, ribonucleotides, alkali, phosphorans, detergents) (Fig. 2b) – revealed close similarity between strains *S. aureus* 1 and 16. Scores for carbohydrates (Fig. 2a) for these strains ranged between −0.5 and 0.2 on axis 1 and between −1.8 to 0.5 for axis 2, while scores for other carbon sources (Fig. 2b) were between −1.0 and 3.0 for axis 1 and −0.4 and −0.1 for axis 2. Scores for strain *S. aureus* 2 were located between −0.5 and 0.5 for axis 1 and between 0.1 and 1.8 on axis 2 for carbohydrates (Fig. 2a), and between −0.5 and 0.7 on axis 1 and −0.1 and 0.9 on axis 2 for other carbon sources (Fig. 2b). These results suggest closer similarity in the physiological properties between *S. aureus* 1 and 16 than *S. aureus* 2.

To confirm these results the three isolates of *S. aureus* were differentiated from each other on the basis of the size of their PCR coagulase gene products and their AluI, EcoRI, HaeII and TaqI restriction digest patterns. The size of the PCR products ranged from c. 720 bp (*S. aureus* 2) to c. 850 bp (*S. aureus* 1 and 16) (Table 2).

Restriction enzyme digestion of the total nested PCR products generated multiple DNA fragments (Table 2, Fig. 3). Digestion with TaqI, EcoRI and HaeIII of the coagulase gene products revealed the same patterns for strains *S. aureus* 1 and 16 in contrast to strain *S. aureus* 2. We did not obtain a positive product after the digestion of the total nested PCR products for *S. aureus* 1 with enzyme AluI but the results obtained with this enzyme for two other strains revealed significant differences in the patterns. The received molecular typing of the *S. aureus* strains examined confirms earlier suggestions about the high similarity between strains 1 and 16 and their differences in comparison with strain 2.
Discussion

For the first time, we describe a system based on CZE as a useful and reliable method making it possible to discriminate between S. aureus isolates. The main benefit of the electrophoretic method is the time needed to gain the results. In our studies, 6–30 min elapsed until the final results were available; it can therefore be considered as almost instant (Buszewski et al., 2003; Kłodzińska et al., 2005, 2006a, b, 2007; Szumski et al., 2005, 2007). This particular quality of the method appears to serve as a revolutionary solution, especially as conventional techniques of microbiological detection often require several days before specific results are known. The identification times have been reduced here to such extent that correct treatment of various infection types becomes available essentially at the initial moment of clinical care. The migration times in CZE also vary among individual bacterial strains.

However, these measurements thus far have not been confirmed by molecular methods. Molecular methods have several advantages over, for example: bacteriophage typing (laborious and of limited utility), plasmid analysis (some isolates do not harbour any plasmids, some plasmids are inherently unstable), and antibiogram profiles (not stable traits, cannot be used reliably for epidemiologic typing). PCR and RFLP of different DNA targets were shown to be specific and highly sensitive and can be easily applied in a microbiological laboratory for differentiating clinical isolates of S. aureus, especially those that do not allow correct identification by commercially available methods (Goh et al., 1992; Grant et al., 1994; Kontos et al., 2003). With the coagulase gene RFLP method, individual strains can be easily compared by both the number of PCR-amplified gene products and the sizes of their restriction enzyme digest fragments. Because of the heterogeneity in the repeated unit-encoding locus, the coagulase gene, as has been shown in many publications (Goh et al., 1992; Schwarzkopf & Karch, 1994; Hooker et al., 1999; Raimundo et al., 1999), is a potential target for type classification on the basis of sequence analysis. Furthermore, nucleotide sequencing here provides an additional source of typing and a highly specific epidemiological marker with high discriminatory value.

We have used the coagulase gene typing method to analyse three S. aureus isolates (1, 2 and 16) isolated from different patients in the same hospital and at the same time. Two of them, S. aureus 1 and 16, were epidemiologically linked to the outbreak and had identical coagulase gene RFLP profiles. Most probably, the single S. aureus 2 strains with different RFLP profile were isolated from a patient who was a S. aureus carrier before hospital admission. The present study as well as others (Goh et al., 1992; Schwarzkopf & Karch, 1994; Raimundo et al., 1999) clearly demonstrates the potential usefulness of this coagulase gene polymorphism.

Fig. 3. Restriction enzymes digest of the PCR coagulase gene products from different strains Staphylococcus aureus (nr 1, 2, 16) isolates.

Table 2. Comparison of Staphylococcus aureus strains (nr 1, 2 and 16) with molecular typing based on coagulase gene DNA fragment length polymorphism

<table>
<thead>
<tr>
<th>Bacterial strain and Accession number</th>
<th>PCR product (approximate bp)</th>
<th>Presence of restriction digest fragment of the following size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AluI</td>
</tr>
<tr>
<td>S. aureus 1 (EU490368)</td>
<td>850</td>
<td>700</td>
</tr>
<tr>
<td>S. aureus 2 (EU490369)</td>
<td>720</td>
<td>680, 400, 350</td>
</tr>
<tr>
<td>S. aureus 16 (EU490370)</td>
<td>850</td>
<td>700</td>
</tr>
</tbody>
</table>

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method for the reliable and simple investigation of nosocomial outbreaks due to either methillicin-resistant *S. aureus* or methillicin-susceptible *S. aureus* infections.

Additional tests, performed during this study and carried out by CA analysis of physiological properties of three bacterial strains (*BIOLOG*), confirmed the results received by molecular methods and revealed a close physiological connection of isolates *S. aureus* 1 and 16.

As visualized in our data, the sensitivity and the specificity of the CZE method, which was for the first time used for the differentiation of *S. aureus* isolates, appear to be very high. The results observed in the analysis of various strains of the same bacterial species, *E. coli*, support this notion (Kłodzińska et al., 2006a).

Analyses of mixtures containing various species of bacteria may pose a problem, because the initial data indicates that the cells can influence each other's mobility in the system. Further studies incorporating more species of bacteria are needed in order to adjust the method for these variables. If a reliable electrophoretic database can be created, the CZE method will be very useful in the identification of different bacterial strains as a screening method for medical diagnosis. If necessary, additional tests can be used under such circumstances, particularly methods based on indirect analysis detecting the presence of infection through immunological assays. The future lies in coupling of electromigration techniques with two-dimensional (2D) electrophoresis (Raimundo et al., 1999; Wulfkuhle et al., 2001; Vijayendran et al., 2007). Our newest approach for resolving an increased number of protein components in eukaryotic proteomes is prefractionation of samples before 2D polyacrylamide gel electrophoresis (PAGE) using CZE. Success in using this approach depends on the creation of a electrophoretic bacterial reference library, which would allow data (migration times, electrophoretic mobilities and UV-VIS spectra) to be compared against the reference material and final analysis of fractionated bacterial cells using 2D PAGE (comparison of protein obtained from different bacterial strains). The results of the initial experiments indicate that diagnostics based on this technology can be extremely sensitive, inexpensive and, primarily, very fast.

**Conclusions**

The CZE typing of the *S. aureus* strains examined clearly revealed differences and similarities between these strains, and these were confirmed via physiological and molecular investigation. These results give sufficient and real grounds to conclude that CZE could be a novel, fast and cheap method of identification and typing of bacterial strains. However, future investigation is necessary to improve this method and create a working database.

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**References**


