Epidemiologic Genotyping of Methicillin-Resistant Staphylococcus aureus (MRSA) by Pulsed-Field Gel Electrophoresis (PFGE)

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Abstract

Staphylococcus aureus has long been recognized as one of the leading cause of hospital infections all over the world. Increased frequency of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitalized patients and possibility of vancomycin resistance requires rapid and reliable characterization of isolates and control of MRSA spread in hospitals. Typing of isolates helps to understand pathogenesis and route of the hospital pathogen spread. In this study, in the analysis of an outbreak of MRSA infections in one surgical ward, we used pulsed-field gel electrophoresis (PFGE) as a method of typing. PFGE revealed one epidemic strain type A in 13 out of 16 patients, and another two types (type B in two patients and type C in one patient). Discussing the typing results in the ward has changed the admission policy of patients with infected vascular ulcers who were then cured as outpatients, and admitted for surgery after that. This policy resulted with the stopping of the outbreak; during next 2,5 year there was no further MRSA outbreak in the ward. PFGE also showed subtypes which enabled the insight into dynamics of MRSA strain changes during the outbreak. PFGE could be recommended as a screening method in the MRSA outbreak analysis. Because of it's high discriminatory power still remains the gold standard for MRSA typing.

KEY WORDS: methicillin-resistant *Staphylococcus aureus*, infection control, pulsed field gel electrophoresis

Introduction

Staphylococci are among the most important causes of both hospital- and community acquired infections worldwide. It is well known that MRSA, like methicillin-sensitive *Staphylococcus aureus* (MSSA) could colonise or infect patients. MRSA strains were not found to be more virulent than MSSA strains and caused the same spectrum of infections (1). *S. aureus* causes superficial and deep skin and soft tissue infections, bacteraemia, endocarditis, osteomyelitis, pneumonia, food poisoning, toxic-shock syndrome and staphylococcal scaled skin syndrome. In the early 1950s, acquisition and spread of β-lactamase-producing plasmids, decreased the effectiveness of penicillin for treating *S. aureus* infections. In 1959, methicillin, a synthetic penicillin, was introduced. However, by 1960, were identified methicillin-resistant *S. aureus*, as a results of *S. aureus's* acquiring the *mec* A gen, which encodes for an altered penicillin-binding protein gen (PBP2a). By the early 1960's, European hospitals were reporting outbreaks of MRSA infections (1,2,3,4,8,9,10,11,12). In Bosnia and Herzegovina, we quoted an increment of MRSA infections by the early 1990's with the beginning of the war. The multidrug-resistant phenotype of MRSA strains and their intrinsic β-lactam resistance make them difficult and costly to cure. Controlling MRSA remains a primary focus of most hospital infections control programs. The aim of this study was to investigate the epidemiological characteristic and clinical outcome of infections caused by MRSA. Bacterial strain typing has become an important clinical tool to investigate suspected outbreaks and to evaluate the route of the hospital pathogen spread. The clonal relationship among MRSA isolates was analyzed by pulsed-field gel electrophoresis (PFGE) (4,5,6,10,11,12,13).

Materials and Methods

Strains

This retrospective study was carried out at one surgery ward. We had 22 strains of MRSA, obtained from 16 patients. The data, we obtained from each patient were: underlying condition, the duration of hospital stay, date of admission and discharge. All patients were with signs of wound infections, so we have taken a wound swabs.

Methods

The strains were identified with current phenotypic methods. The colonies of *S.aureus* on 5% blood agar were 1 to 3 mm in diameter, pigmented yellow, smooth, convex with an entire edge after 24 hours incubation on 37 °C. Identification of *S. aureus* was carried out by production of DNase (2,3,4). All strains were DNase positive. The antibiotic susceptibility testing was performed by the disk-diffusion method, according to NCCLS guidelines. The antimicrobial drugs that tested were: penicillin, erythromycin, azithromycin, gentamycin, trimethoprim-sulfamethoxazole, clindamycin, rifampin and vancomycin. Oxacillin-resistance was determined after incubation for 24 hours at 35 °C with a 1 μg oxacillin disc. Resistance was reported if there was growth within a 10 mm zone of inhibition. MRSA isolates were collected and stored at –20°C until analyzed.

PFGE analysis

DNA isolation

A single colony of *S. aureus* was inoculated into 5 cm^3 of brain-heart broth and incubated at 37 °C for 18 to 24 hours. Broths culture (0,6 cm^3) of *S. aureus* was centrifuged at 7000 rpm for 2 minutes, supernatant was aspired, the pellet was resuspended in 1 cm^3 autoclaved Tris/EDTA/NaCl (TEN) buffer twice, and was centrifuged again. The resuspended cells was rinsed in 300 μL autoclaved EC buffer. After that, 2 mm^3 lysostaphin stock solution were added and briefly vortexed. Afther that, bacterial-lysostaphin suspension was mixed with 300 mm^3 low melting temperature agarose (1,7% agarose in EC buffer at 56 °C), briefly vortexed and quickly dispensed in small disposable mold (100 mm^3 each). The plugs were allowed to solidify at room temperature for 10 minutes. The plugs were removed and placed into a tube containing 3 cm^3 EC buffer and incubated at 37°C for 1 hour. After that the plugs were washed three times in 3 cm^3 TE buffer and then were stored at 4 °C.

Sma I restriction enzyme digestion and gel electrophoresis condition

The plugs were washed in TE buffer twice, before macrorestriction with 30 IU Sma I for 3 hours at room temperature. After that, plugs were set up in agarose gel 1% and suffused with 0,7% LMP agarose. The DNA macrorestriction fragments were separated in agarose gels by PFGE using a contour –clamped homogenous electric field apparatus, i.e. CHEF DR III System (Bio-Rad, Hercules, California, USA). Running parameters were as follows: strength 600 V/m; temperature 14 °C; initial time 5 s; final time

40 s; and duration 20 h. The standard λ concatamer (50-500 Kbp, Roche Diagnostics) was used as molecular size marker (14,15,16,17,18,19,20,24).

Visualisation

After the electrophoresis run was completed, the gel was stained in 1 μg/cm^3 ethidium bromide solution for 20 minutes in a covered container and distained in fresh distilled water for 10 minutes. Gels were photographed under UV light.

Data analysis

Percentage of similarity was identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance was 3%, and optimization was 0,5%. A similarity coefficient of 80% was selected to define the pulsed-field type clusters.

Results

An examination of medical staff for carrier status

An examination was performed twice, during the period of an outbreak, and there wasn't carriers among medical staff.

Antimicrobial susceptibility

22 strains of MRSA, isolated from 17 patients, have had exactly the same result of antimicrobial susceptibility. All the strains were susceptible to vancomycin and rifampin, and were resistant to penicillin, erythromycin, azithromycin, gentamycin, trimethoprim-sulfamethoxasole, clindamycin and oxacillin.

Pulsed-field gel electrophoresis

During the preparation for typing, one strain (from the patient No 10 from Table 1.) was perished, so we did the genotyping with 21 strains, from 16 patients. From these 21 strains we detected 3 different profiles in the PFGE analysis (Figure 1.). Isolates from 13 patients represent the major type that belongs to pulsotype A (81,2% patients, or 80,9% strains); lanes 1-8, 11-14 and 17 are the pulsotype A. With visual inspection the strain No 4 (Figure 1.b) was different from the other strains checked as type A in intensity of first band. Next another types we marked as type B two patients. No 15 and 16: 12,5% patients, or 9,5% strains) and C (one patient, No 9 with two identical isolates: 5,8% patients, or 9,5% strains). With visual inspection type B was different from the type A in 7 bands; type C was different from the type A in 8 bands, and from the type B in 12 bands.

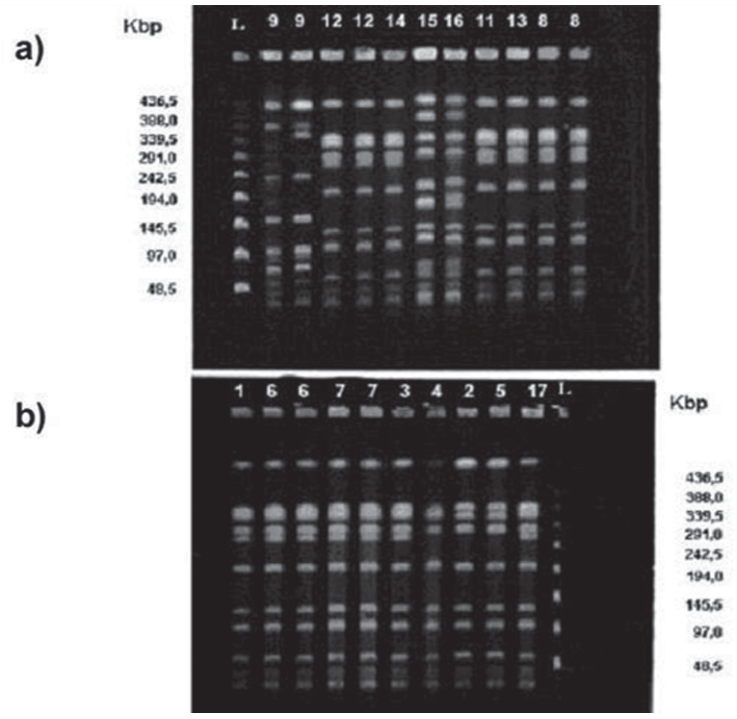

FIGURE 1. a) and b) PFGE patern of MRSA isolates from the patients at surgery ward No 1 – 17: patients; L – λ concatamer; type A: lanes 4-6, 9-12 at Figure 1.a), and 1-10 at Figure 1.b), type B: lanes 7 and 8 at Figure 1.a), type C: lanes 2 and 3 at Figure 1.a) Kbp: kb pairs

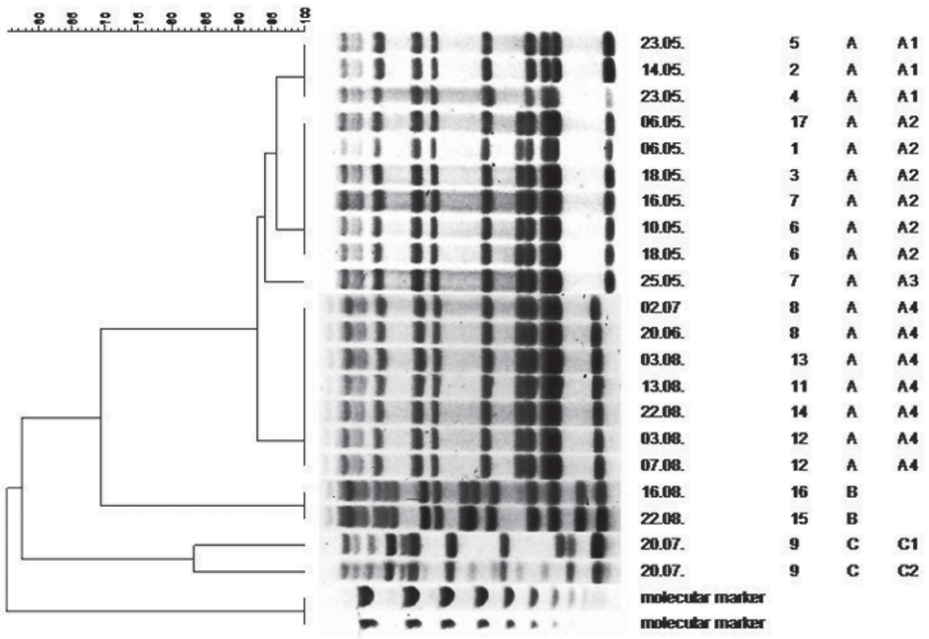

FIGURE 2. Classification of types, description of similarities of 21 MRSA strains isolated from 16 patients with PFGE
First line: date of isolation; second line: patient's number from the Table 1.; third line: pulsotype of strain; forth line: subtype of strain

The Figure 2, shows dendrogram of similarities of analyzed MRSA strains. On the bottom are bands of λ-concatamer

As we previously have said, a similarity coefficient of ≥ 80% was selected to define the pulsed-field type clusters. That type was signed as types A, B and C. As we have said in description of Figure 1, with visual inspection, we couldn't see the differences between the lanes of type A, except the intensity in first band in the strain No 4. Computer comparison, as we can see on the Figure 2., showed that within type A exist 4 subtypes. Among them, type A1 (strains NO 5, 2 and 4) and type A2 (strains NO 17, 1, 3, 7, and 6) showed a high degree of similarity 95%, A1 and A3 (another strain NO 7) had 93% similarity, and A1 and A4 (strains No 8 (both), 11, 12 (both), 13 and 14) had 92% similarity. The strains of subtypes A2 and A3 were isolated from the same patients in the distance of 9 days, and the detail of their comparison shows the Figure 3. That two strains are distinguished in one band (that shows the Figure 3), but it is possible that is only a difference in density. Namely, the similarity between those two strains is 96%. Both strains B were identical. Subtypes of strain C (C1 and C2) are related only 83%, therefore they are on the border of two types, but we couldn't see that with visual inspection (Figure 1).

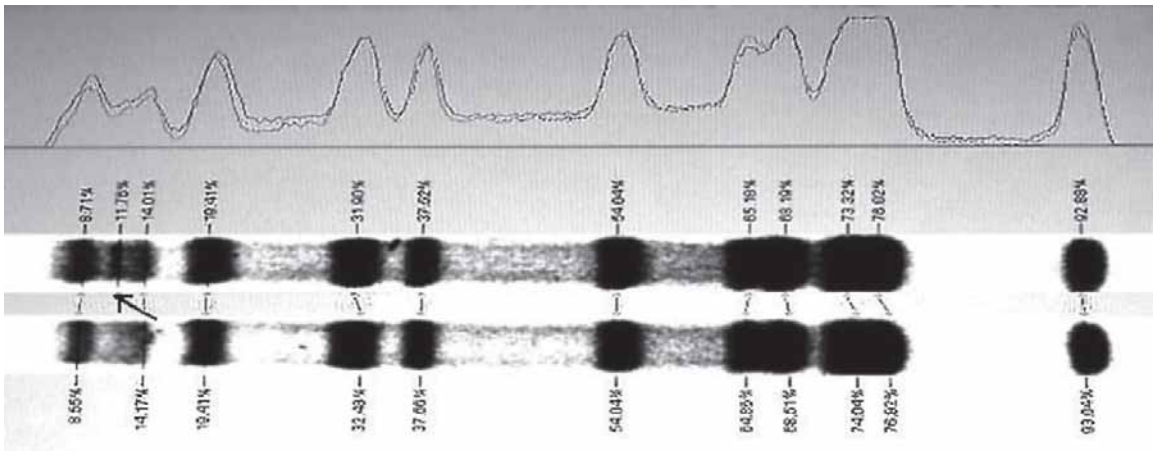

FIGURE 3. Comparison of PFGE patterns of strains isolated from patient NO 7 (A2 and A3 subtype). The arrow marks a new band.

Patient No	Diagnosis	Days in hospital	Date of admission and dismission	Type
1.	Insufitientio circulatoria extr. inferioris sin., St post amputationem cruris dex.	32	09.04.-11.05.	A (A2)
2.	Gangraena incipiens sin.	35	02.05.-06.06.	A (A1)
3.	Gangraena pedis dex.	19	03.05.-22.05.	A (A2)
4.	Syndroma Leriche	19	03.05.-22.05.	A (A1)
5.	Atherosclerosis obliterans arteriae femoralis dex., St. post by-pass femoro-popliteris sin.	16	09.05.-25.05.	A (A1)
6.	Gangraena pedis dex., St. post amputationem cruris sin.	18	11.05.-29.05.	A (A2),A(A2)
7.	St.post amputationem femoris sin., dehiscetio vulneris operationem, Diabetes mellitus	32	14.05.-15.06.	A (A2),A(A3)
8.	Stenosis arteriae iliacae dex.	56	14.05.-09.07.	A(A4),A(A4)
9.	Syndroma Leriche, Diabetes mellitus	48	14.06.-01.08.	C(C1),C(C2)
10.	Gangraena diabetica digiti III pedis dex.	19	22.06.-16.07.	Strain perished
11.	Gangraena incipiens hallucis dex., St.post amputationem femoris sin.	39	14.07.-22.08.	A (A4)
12.	Gangraena pedis dex., Diabetes mellitus	34	17.07.-20.08.	A (A4)
13.	Gangraena incipiens pedis dex., Diabetes mellitus	14	24.07.-07.08.	A(A4)
14.	Syndroma Leriche, Diabetes mellitus	36	07.08.-12.09.	A (A4)
15.	Occlusio arteriae popliteae sin., Diabetes mellitus	21	07.08.-28.08.	B
16.	St.post amputationem femoris sin. et exarticulatio coxae dex.	13	16.08.-29.08.	B
17.	No data		06.05.	A (A2)

TABLE 1. Characteristics of the patients included in the investigation

Analysis of an outbreak

Characteristics of the patients included in this investigation, were showed in the Table 1.: diagnosis, days in hospital, date of admission and discharge, and the type of isolated strain MRSA. The strain of the patient No 10 was perished, so we couldn't analyze it. We couldn't get the data about patient No 17, but we have had a strain, and the date of isolation, so we included it in our investigation. As we can see from the Table 1, all our patients have had a problems with vascularization of extremity, till the gangrene. Some of them were amputated. The length of hospitality was between 13 and 56 days, with a middle value of 26,5 days, and median of 28,2 days. During the outbreak, a standard measures against the spreading of MRSA were undertaken on the ward, without success: new cases were appearing continued. After we have done a PFGE typing, we had a discussion with whole staff in the ward and showed them a Figure 1. and 2. and a leader has changed a admission's policy on the ward. During the outbreak, patients with infected vascular ulcers were admitted on the ward and there cured until the operation. After that, the patients like that, were cured as an outpatients and than admitted for a surgery. This policy resulted with the stopping of the outbreak; during next 2,5 year there was no further MRSA outbreak in the ward.

Disscusion

S. aureus is a major nosocomial pathogen that causes a range of diseases, including endocarditis, osteomyelitis, pneumonia, toxic-shock syndrome, food poisoning, carbuncles, boils and infection of surgical wounds. Increased frequency of methicillin-resistant *S. aureus* (MRSA) or multidrug-resistant phenotype of MRSA strains and their intrinsic beta-lactam resistance, make them difficult and costly to treat. Bacterial strain typing, or subtyping, has become an important clinical tool to investigate suspected outbreaks and to evaluate nosocomial transmission (20). Numerous techniques are available to differentiate MRSA isolates. Historically, isolates were distinguished by phenotyping methods, but they have limitations, because many *S. aureus* isolates are non-typable by them. Genotyping methods have significant advantages because they are focused on DNA-typing. In this study, in the analysis of an outbreak of MRSA infections in one surgical ward, we used pulsed-field gel electrophoresis (PFGE) as a method of typing. PFGE was first described by Schwarz and Cantor, and it is a highly discriminatory method for the differentiation of staphylococcus (18). This method uses infrequent-cutting restriction enzymes to digest extracted genomic DNA into about 10 to 20 fragments. This fragments are in range between 10 and 800 kilobase pairs (kb), and they are too large to be separated by conventional gel electrophoresis. In PFGE the orientation of the electric field across the gel is periodically changed (pulsed), allowing DNA fragments on the order of megabase pairs to be effectively separated, according to size. All bacterial species can theoretically be typed by PFGE, results are highly reproducible and method is harmonized recently (21). PFGE requires intact genomic DNA, and special care must be taking during the isolation of DNA. To order to avoid the risk of mechanical breakage of DNA molecules during the extraction procedure, each sample is incorporated into low melting point agarose, thus protecting the DNA, and at the same time allowing the free flow of solutions necessary for cell wall lysis and enzymatic digestion of cell proteins. The isolated DNA is than submitted to digestion with restriction endonucleases that recognize few sites along the chromosomes. The agarose plugs containing the digested DNA are than submitted to the PFGE. PFGE has been used for investigation MRSA and has been compared with the other typing methods in many studies. A great number of restrictions enzymes have been tested, but no one has shown better performance than Sma I. All isolates are typable and re-

producible, even after extensive sub-culturing. The discriminatory power is equal to or superior to phenotypic techniques as well as to genotypic techniques such as RAPD, PCR-RFLP and rep-PCR. Due to aforesaid, PFGE has many of the characteristic attributed to an ideal typing technique and has been proposed as the gold standard for MRSA typing. However, there are limitations for the use of PFGE, such as a long time interval until the final result are obtained and specialized equipments used for this technique (14,15,16,17,18,19,20,22,23,24). Interpretation of obtained results is standardized by the interpretations scheme which proposed Tenover *et al* (14). Using this scheme, isolates that poses the same PFGE profile are considered as being identical. Isolated that differ by a single genetic event, reflected by a difference in one to three bands, are considered as being probably related. Isolates that differ in four to six bands, representing two independent genetic events, are considered as being possibly related, and isolates possessing a difference in more than six bands are considered as being unrelated. It is important to highlight that such criteria are applicable only to the analysis of a small number of isolates obtaining during epidemiological studies of outbreaks in hospitals or communities during a relatively short period of time (a few months), as we had in our study. In our study PFGE has showed completely effectiveness.

Conclusion

Antimicrobial susceptibility, as a phenotyping method is the simplest method for performing, and it's results are simple to interpret, but it has a limitations, because many *S. aureus* strains are non-typable by it. PFGE is considered the gold standard technique for MRSA typing due to its high discriminatory power, its excellent reproducibility, and its good correlation with epidemiologically linked data. PFGE is a slow and time-consuming procedure that requires specifically trained personnel and sophisticated equipment, but in our study has shown completely effectiveness.

List of Abbreviations

DNA	-	deoxyribonucleic acid
DNase	-	deoxyribonuclease
EC buffer	-	a kind of buffer
EDTA	-	ethylenediaminetetraacetic acid
LMP	-	low melting point
MSSA	-	methicillin-sensitive *Staphylococcus aureus*
MRSA	-	methicillin-resistant *Staphylococcus aureus*
NCCLS	-	National Commitee for Clinical Laboratory Standards
PBP	-	penicillin binding protein
PCR	-	polymerase chain reaction
PFGE	-	pulsed-field gel electrophoresis
PVL	-	Panton Valentin-leukocidin
RAPD	-	randomly amplified polymorhic DNA analysis
Rep-PCR	-	repetitive DNA sequence PCR
RFLP	-	restriction fragment lenght polymorphism
RNA	-	ribonucleic acid
SCC-mec	-	staphylococcal chromosomal cassette mec
Sma I	-	Serratia marcescens I restriction enzyme
TBE buffer	-	Tris base+boric acid+EDTA buffer
TE buffer	-	Tris-Cl+EDTA buffer
TEN buffer	-	Tris/EDTA/NaCl buffer

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