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EXPERIMENTAL / LABORATORY STUDIES

Typing of Methicillin-Resistant *Staphylococcus aureus* by Ribosome Spacer and Arbitrarily Primed Polymerase Chain Reaction

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Abstract: Thirty-five methicillin-resistant Staphylococcus aureus (MRSA) clinical isolates were collected from 3 hospitals in the northern and southern parts of Palestine between February and May 1998. These isolates were typed by ribosome spacer PCR (RS-PCR) and arbitrarily primed PCR (AP-PCR). RS-PCR generated 9 different genotypes. The use of AP–PCR provided a high resolution typing method and allowed us to define 11 different clusters. Three major clusters, however, based on the combination of both typing methods, spread throughout the neonatal and intensive care units of Rafidya Hospital during the entire period.

Key Words: Staphylococcus aureus, epidemiology, polymerase chain reaction

Introduction

Since its first identification in the early 1960s, methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major pathogen involved in hospital-acquired infections in many parts of the world (1-6). Increased surveillance, including screening of high-risk patients, has been recognized as an important component of effectiveness hospital infection control programs. Therefore, rapid and accurate identification of MRSA is essential. A variety of phenotypic, and more recently genotypic, techniques have been employed for strain typing (7). The recent development of DNA-based techniques has reduced the dependence on phenotyping. Genotyping of bacterial strains is based on the principle that epidemiologically related isolates have genetic features that distinguish them from other epidemiologically unrelated strains (8-11). Amplification of the 16S-23S rRNA intergenic spacer region by the ribosome spacer PCR (RS-PCR) can detect a significant level of length variation that is useful in genotyping at a subspecific level. Arbitrary primed PCR (AP-PCR) results in epidemiological typing of MRSA outbreaks are comparable with those obtained by pulsed field gel electrophoresis (PFGE) (12-13). In this study, we characterized the MRSA isolates obtained from 2 different geographical areas in Palestine using 2 PCR typing methods (RS-PCR and AP-PCR) and assessed the correlation among the results yielded by these methods. We also described the dissemination of MRSA isolates in Rafidya Hospital and investigated the possible reasons for their spread.

Materials and Methods

Clinical Specimens

MRSA isolates. A total of 35 MRSA isolates, 32 from Rafidya Hospital in northern Palestine and 3 from 2 other hospitals in southern Palestine, were collected between February and May 1998. Information about the origin of these isolates is presented in Table 1. Isolates were recovered from patients with infection (10 isolates), colonized patients (12 isolates) and the hospital environment (13 isolates). Isolates that caused infections were obtained from the umbilicus (4 isolates), urine (2 isolates), wounds (2 isolates) and prostate (1 isolate). *Strain Identification.* Isolates were identified as *S. aureus* according to colonial and microscopic morphology, positive catalase and coagulase production by Staphytect plus tests (Oxoid).

Determination of methicillin resistance

The isolates grown on culture plates were identified as MRSA following the National Committee for Clinical Laboratory Standards guidelines (14).

Preparation of Bacterial DNA

Five to 10 colonies were suspended in 100 µl of lysing solution containing 125 µg/ml lysostaphin (Sigma), and 1 mg/ml lysozyme (Sigma) in TE buffer. The suspension was incubated at 37 ºC for 60 min, 10 µl of (10 mg/ml) proteinase K (Sigma) was then added and incubation continued for an additional 30 min. The suspension was heated at 97 ºC for 10 min and centrifuged, and the supernatant was stored at –20 ºC for amplification.

RS-PCR Amplification Procedure

PCR was performed in 25 µl volume, with 2.5 µl of 10X PCR buffer (500 mM KCl, 100 m MTris HCl) [pH 9.0], 250 mM each of dATP, dCTP, dGTP and dTTP, 2 µl of 25 mM $MgCl_2$ and 1 U of *Taq* polymerase (Promega). Two primers were included in the typing assays G1 5′-GAAGTCGTAACAAGC-3′ and L1 5′-CAAGGCATCCACCGT-3′ (13). Amplification was performed as follows: 2 min at 94 ºC, followed by 34 cycles for 1 min at 94 ºC, 1 min at 45 ºC (annealing), and 1 min at 72 ºC (elongation) and finished with an additional 5 min extension step at 72 ºC.

AP-PCR Amplification Procedure

AP-PCR was performed in a 25 µl reaction mixture with 2.5 µl of 10X PCR buffer containing 500 mM KCl, 100 m MTris HCl [pH 9.0], 250 mM each of dATP, dCTP, dGTP and dTTP, 2 µl of 25 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase (Promega). The primer included in the assays was P7, 5'-CAA GGC ATC CAC CGT-3' (15).

Amplification was performed as follows: 4 min at 94 ºC, followed by 35 cycles of 1 min at 94 ºC, 1 min at 42 ºC, and 1 min at 72 ºC. The program finished with an additional 10 min extension step at 72 ºC.

The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide. The DNA was visualized on a UV transilluminator, and photographed.

Banding patterns that appeared identical in terms of size and number of bands were considered to represent the same strain, which was designated in Latin numerals (I, II, III, etc...) for RS-PCR patterns and lower case letters (a, b, c, etc...) for AP-PCR.

Results

RS-PCR. The RS-PCR assay of MRSA isolates yielded amplicons that ranged in size from 300 bp to 800 bp, with 1 to 6 resolved fragments per isolate. Nine RS-PCR patterns identified were designated I through IX. The most frequent patterns were I (8 isolates) and II (5 isolates). The remaining patterns were represented by 1-4 isolates (Figure 1).

AP-PCR. DNA amplification using P7 arbitrary primer resulted in 11 different DNA banding patterns. The number of amplified DNA fragments ranged between 1 and 11 distinct bands in each pattern with a size range of 300-900 bp. Pattern a was represented by 12 isolates. The remaining patterns were represented by 1-4 isolates (Figure 2).

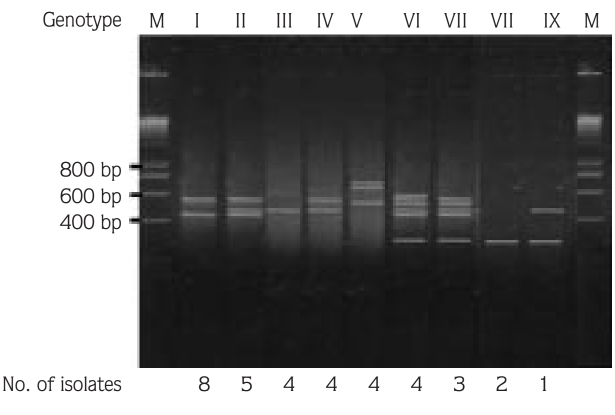

Figure 1. RS-PCR patterns of MRSA isolates. Lane M, molecular size markers (100-bp ladder DNA), some of the molecular sizes of the marker are indicated on the left.

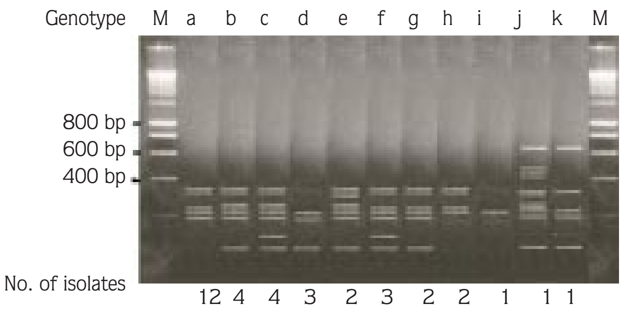

Figure 2. AP-PCR patterns of MRSA isolates. Lane M, molecular size markers (100-bp ladder DNA), some of the molecular sizes of the marker are indicated on the left.

In this study, 6 isolates (A5, A11, C37, R68, R69, and R95) were considered epidemiologically unrelated because either they originated from different geographic areas (n = 3), or they had been sampled from recently admitted patients with no link to other known MRSA carriers and/or infected patients in the hospital (n = 3) (Table). These isolates were used as a control for the PCR assay in our study.

Cluster Analysis. The combination of the RS-PCR and AP-PCR patterns define 24 clusters. Of these, 20 clusters were observed among the epidemiologically related isolates of Rafidya hospital (Table 1). The high degree of

Table. RS-PCR and AP-PCR genotypes of MRSA.

Isolate[a]	Source		RS-PCR	AP-PCR	Cluster type
	Site	Ward[b]			RS:P7
R 715	Environment	ICU	III	a	III::a
R 313	Environment	ICU	III	a	III::a
R 095	Blood	Outpatient	VI	c	VI::c
R 588	Environment	ICU	VI	c	VI::c
R 519	Environment	NU	VIII	e	VIII::e
R 464	Groin	NU	II	f	II::f
R 760	Nose	ICU	II	f	II::f
R 316	Environment	NU	I	a	I::a
R 442	Environment	NU	I	a	I::a
R 730	Nose	ICU	I	a	I::a
A 005	Urine	ND	VIII	d	VIII::d
R 534	Environment	NU	VI	e	VI::e
R 578	Environment	NU	I	h	I::h
R 271	Umbilicus	NU	II	a	II::a
R 763	Umbilicus	NU	II	a	II::a
R 347	Groin	NU	II	a	II::a
R 272	Nose	NU	V	c	V::c
R 561	Prostate discharge	HCW	V	c	V::c
R 069	Pus	Outpatient	I	b	I::b
R 386	Nose	ICU	III	d	III::d
C 037	Umbilicus	ND	V	j	V::j
R 479	Umbilicus	NU	III	a	III::a
R 731	Groin	NU	IV	b	IV::b
R 322	Environment	ICU	IV	i	IV::i
R 668	Environment	NU	VII	a	VII::a
R 669	Environment	ICU	I	f	I::f
A 011	Urine	Outpatient	V	k	V::k
R 756	Nose	NU	VII	g	VII::g
R 422	Environment	NU	I	d	I::d
R 647	Nose	ICU	IX	a	IX::a
R 639	Environment	NU	IV	g	IV::g
R 769	Groin	NU	VII	a	VII::a
R 655	Nose	NU	VI	b	VI::b
R 068	Pus	Outpatient	I	b	I::b
R 727	Nose	ICU	IV	h	IV::h

[a] R, Rafidya Hospital (Northern Palesine); A, Alia; C, Caritas (Southern Palestine).
[b] NU, Neonatal unit; ICU, Intensive care unit.
HCW, Health care worker; ND, not determined

heterogeneity should be considered together with the clear evidence of 3 major clusters (I::a, II::a and III::a) including 3 isolates each. Two isolates of cluster I::a were collected from the environment of the neonatal unit, while the other was collected from a colonized patient in the intensive care unit. Isolates of cluster II::a were collected from colonized patients in the neonatal unit. Two isolates of cluster III::a were found in the environment of the intensive care unit, and the third isolate was collected from an infected patient in the neonatal unit. Together, clones I::a, II::a and III::a represented 26% of all MRSA isolates. The remaining clones were found only in a small number of isolates (1 or 2 isolates).

Discussion

Bacterial strain typing, or subspeciation, has become an important clinical tool to investigate suspected outbreaks and to evaluate nosocomial transmission. MRSA has emerged worldwide as an important nosocomial pathogen. In some US hospitals, MRSA already accounts for 30% to 50% of all nosocomial *S. aureus* isolates. The situation is comparable in many European centers (3-4,16-17). The proportion of MRSA compared to all nosocomial *S. aureus* isolates studied was > 50% in Portugal and Italy and > 30% in Turkey and Greece. The methicillin-resistance rate was low in the Netherlands (2.0%) (5) and in Switzerland (1.8%) (3-4). In the Scandinavian countries, methicillin-resistant strains still account for <1% of all nosocomial *S. aureus* isolates (3). Methicillin resistance has become a serious problem in Palestine, but is still relatively low compared to those cases that have been reported in many other countries (3-4,16-17). The prevalence of methicillin resistance among *S. aureus* was 11% (44/401) (18). An attempt was made to study the epidemiology of 35 MRSA isolates obtained from 2 different geographical areas in Palestine using RS-PCR and AP-PCR. This study is the first molecular epidemiological study in Palestine. RS-PCR generated 9 distinct patterns among the MRSA isolates. The most prevalent spacer types were types I and II represented by 23% and 14% of the isolates, respectively. Of the 29 epidemiologically related isolates of type I, 4 isolates were from the neonatal unit environment and the rest were from the environment and colonized patients in the intensive care unit of Rafidya Hospital (1 each). Type II isolates were obtained from the colonized patients in both the neonatal and intensive care units.

AP–PCR provided a high resolution typing method and allowed us to define 11 different DNA banding patterns. The most prevalent type was type a, represented by 34% of the isolates. The isolates of this type were found in the environment and colonized patients in both the intensive care and neonatal units of Rafidya Hospital.

The recovery of RS-PCR and AP-PCR major clusters in the majority of the isolates of neonatal and intensive care units, respectively, is indicative of typical nosocomial outbreaks and cluster spread. The high density of the neonate population (the neonatal unit of Rafidya Hospital is a single large room with 13 incubators) and frequent close contact from physicians and nurses, during which hand washing and asepsis may be overlooked, promote horizontal transmission of MRSA strains.

Combinations of RS-PCR and AP-PCR resulted in 3 major clusters (I:B:a, II:C:a; 3 isolates each). The recovery of these clusters in the majority of the isolates of neonatal and intensive care units, respectively, agrees with the previous conclusion that the probable common source of the MRSA strains was the neonatal unit. Thus, the demonstration in these units of an evident MRSA cluster spread should provide important motivation to reinforce routine infection control procedures to limit horizontal transmission of MRSA. Moreover, detection of clusters (I::b and VI::c) in the epidemiologically unrelated isolates suggests that these strains may emerge as a public health problem in Palestine.

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