REPRODUCIBILITY, STABILITY AND DISCRIMINATORY POWER OF RESTRICTED ENZYME ANALYSIS OF PLASMID DNA AMONG METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

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SUMMARY

Methicillin-resistant Staphylococcus aureus (MRSA) is a significant epidemiological problem. Detecting the sources of epidemic strains and preventing their access to patients, however, depend upon the availability of techniques to reliably distinguish among MRSA strains. Molecular typing of MRSA generally demonstrates improved discriminatory power compared with assays of phenotypic traits, and it is frequently preferred.

Objective: Evaluation of the discriminatory power and stability of restricted enzyme analysis of plasmid (REAP) DNA among MRSA strains.

Material and Methods: 54 sequential MRSA isolates from 18 patients collected within 30-36 days (three isolates from every patient, in average 15 days interval between each isolate). Isolates were subjected to plasmid screening; isolates with positive plasmid underwent typing by REAP using Hind III restriction enzyme. Comparison was done to detect plasmid stability and discriminatory power among sequential MRSA isolates.

Result: Among 54 sequential MRSA isolates; 45 were plasmid-positive isolates from 15 patients by plasmid screening gel. Patient's plasmid digestion profile remained unchanged over a period up to one month except in two cases (4.4%). In one case, two distinctly different digestion profiles were detected from a patient (between the initial isolate and the second successive isolate). In the other case, two distinctly different digestion profiles were detected from a patient (between the initial isolate and the third successive isolate). Nine (16.6%) out of 54 MRSA isolates from 3 patients lacked plasmid.

Conclusion: The frequency of genetic alteration in plasmid among MRSA strains in vivo is infrequent and REAP profile of MRSA isolates is very stable in vivo. The diversity and stability of MRSA plasmid types make them excellent epidemiological markers.

INTRODUCTION

MRSA is well known as a nosocomial pathogen that has spread worldwide since it was first described in the early 1960s (Jevons, 1961). Since then MRSA strains have largely been confined to hospitals and long-term care
facilities with steady increase in incidence of infection, which complicate treatment of patients, prolong hospitalization, increase cost and maybe life threatening (Vandenesch et al., 2003). The differentiation between community-acquired MRSA (CA-MRSA) and hospital-associated MRSA (HA-MRSA) is becoming difficult since CA-MRSA could spread into hospitals (Wannet et al., 2004). CA-MRSA causes primary skin infections, mainly furunculosis and abscesses, but can also cause necrotizing tissue infections and fulminant pneumonia in young and previously healthy individuals (Gillet et al., 2002, Reichert et al., 2005). Transmission of MRSA between patients receiving health care and family members and case reports of intra familial spread of CA-MRSA have been described (Wagenvoort et al., 2005).

So eradicating the source of MRSA or interrupting their path to patient are important goal. As MRSA often colonizes hospital personnel, attaining this goal depend upon techniques for characterization epidemic and distinguishing it from endemic MRSA (Wannet et al., 2004).

Phage typing and antibiotyping are not appropriate to trace such strains, not only because of the diversity observed among strains in the same genotype but also because some strains having the same phage type and/or antibiotype belong to distant genotypes. However, at the beginning of an outbreak in a hospital, these two phenotypic markers may be useful to screen closely related strains. (Galdbart et al., 2000).

There are various molecular typing methods available for S. aureus, of them REAP. The frequency of genetic alteration among MRSA strains in vivo and whether plasmid DNA alteration are more common than genomic DNA alteration are yet unknown (Liassine et al., 2004). Multilocus sequence typing (MLST) is used to identify clones within populations of pathogenic microorganisms and genetic variation is observed by assigning the alleles at each locus directly by nucleotide sequencing the internal fragments of seven housekeeping genes. A major advantage of MLST is the ability to compare sequence data between laboratories via the MLST website on the Internet (Berglund et al., 2005).

Pulsed field gel electrophoresis (PFGE) is still the gold standard method for investigating outbreaks and local epidemiology. However, PFGE has major disadvantages, including time consuming, expensive equipment and difficulties in comparing the results of different laboratories (Reichert et al., 2005).

MATERIALS AND METHODS
Bacterial isolates. 54 MRSA clinical isolates were obtained during the period of November through December 2004. The isolates were collected from different infection sites of 18 patients hospitalized in different wards at Benha University Hospital. All patients were critically ill and needed prolonged hospitalization. Data of patients and samples are summarized in table (1). All patients were under antibiotic treatment at the time of samples collection. Three successive isolates were obtained from each patient at various time (12 to 18 days interval) during their hospitalization. All patient isolates of MRSA were identified by conventional means (Kloos, and Lambe1991).

Strains from all sources were tested for coagulase reaction and methicillin and oxacillin susceptibilities before their use in the study. The antibiotic susceptibilities were determined by the Kirby-Bauer disk diffusion method (Bauer et al., 1966). Bacterial isolates were inoculated into 10% skimmed milk and frozen at -70°C until tested.

Plasmid isolation. Plasmid DNA was isolated from the cells by a modification of the alkaline lysis method (Zuccharelli et al., 1990). Cells were grown in 30 ml of L broth overnight in an incubated shaker at 37°C, harvested, and washed once with 1.0 ml of TE buffer (10 mM Tris [pH 7.5], 0.1 mM disodium EDTA). All subsequent manipulations were performed in 2.0-ml microcentrifuge tubes. The cells were suspended in 0.2 ml of TE buffer containing 50ug of lysostaphin (Sigma, Aldrich, Germany) per ml and incubated for 30 min at 37°C. To this, 0.4 ml of 0.2 M NaOH-1% (wt/vol) sodium dodecyl sulfate was added. After 10 min on ice, 0.3 ml of 3 M potassium-5 M acetate was added, and the lysate was cooled for another 10 min on ice. The mixture was centrifuged at 12,000 xg for 10 min at 0°C, and the supernatant was extracted once with buffer-saturated phenol and twice with ether. The preparation was incubated with 2ul of RNase A (10 mg/ml; Oxoid, England) for 15 min at 37°C. After an extraction with 1.0 ml of phenol-chloroform (1:1), the DNA was precipitated with 3 volumes of ethanol. The precipitate was collected by centrifugation, washed once with cold 70% (vol/vol) ethanol, the resultant pellet was dissolved in 80 ul of distilled water and maintained at 4°C until use.

Plasmid screening: Ten microliter of each sample and 5 ul of loading dye were electrophoresed in 0.7% agarose gels in Tris-borate buffer at 100 V for 60 to 90 min, followed by ethidium bromide staining and photography under UV light.

Restriction enzyme digestion. For REAP DNA, 15 ul plasmid-positive preparations were incubated for 3h at 37°C with 2 ul of restriction endonuclease (RE) enzyme Hind III and buffer as recommended by the manufacturer (Sigma,Aldrich.Germany) and transferred to wells in a 0.7% agarose gel in
Tris-borate buffer. A diluted 1-kilobase (kb) DNA ladder (Bethesda Research Laboratories) with running dye was added to at least one well of each gel, and electrophoresis was run in room temperature overnight at 20 to 25 V in Tris-borate buffer. After staining for 30 min with 0.5μg of ethidium bromide and photography, lanes were examined for the number of bands between 1 and 12 kb, and the molecular size of each band was estimated.

**Analysis of REAP differences among sequential patient isolates.** Differences in REAP typing results for each patient’s sequential isolates were divided into categories that were based on assessment of REAP gels. Related REAP types were defined as isolates demonstrating a coefficient of similarity (CS) equal to or exceeding 0.85. The CS was calculated as follows: CS = 2x (number of matching bands)/total number of bands in both strains. REAP types considered to be related if they did not have new or absent bands on the plasmid-screening gels and had only one to three band differences on the REAP gels.

**RESULTS**

**Patients and bacterial isolates.** This study was conducted on 54 MRSA isolates from 18 inpatients. Sources of samples is summarized in table 1

**Table (1): Data of patients and samples**

<table>
<thead>
<tr>
<th>Source of samples</th>
<th>Number of patients</th>
<th>Male</th>
<th>female</th>
<th>children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurosurgery unit</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Intensive care unit</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Burn unit</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Orthopedic unit</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Surgical unit</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Plasmid detection**: Three patients out of 18 (9 isolates out 54 isolates) did not have detectable plasmid. Fifteen patients with 45 MRSA isolates have one or more detectable plasmids as in table 2.

**Table (2): Number of plasmid in MRSA isolates**

<table>
<thead>
<tr>
<th>No. of plasmids in the isolates</th>
<th>No of patients</th>
<th>No. of isolates</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>9</td>
<td>16.7</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>33</td>
<td>61.1</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>12</td>
<td>22.2</td>
</tr>
</tbody>
</table>

**Typing by REAP.** A total of 45 isolates containing plasmids were typed by REAP, all isolates were susceptible to RE. Four types were discriminated by REAP from the 15 initial patient isolates. Another 2 types were observed from 2 patients sequential isolates (Table3). Thirteen patients with 39 sequential isolates did not have different types by REAP, whereas 2 patients with 6
sequential isolates (3 from each patient) had additional two REAP types. The numbers of isolates per patient were (3 isolates) and the interval between subsequent isolates per patient was 15 days in average. One patient had different type during the first 15 days, and another patient had different type during the subsequent 15 day interval. According to CS; no related REAP types were defined.

Table (3): REAP DNA profiles of MRSA isolates.

<table>
<thead>
<tr>
<th>REAP profile</th>
<th>No. of patient</th>
<th>%</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>26.7</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>53.3</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>13.3</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>6.7</td>
<td>3</td>
</tr>
<tr>
<td>*5</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>**6</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

REAP profile no. 1-4 are the profile of the initial MRSA isolates. No.5 is the profile of a patient had different REAP profile during the first 15 days, no.6 is a profile of another patient had another different REAP profile during the subsequent 15 day interval. These profiles was from isolates which gave profile No. 2 and 3 respectively on their previous isolation.

Table (4): Patients and MRSA isolates.

<table>
<thead>
<tr>
<th></th>
<th>Without REAP difference</th>
<th>with REAP difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patient</td>
<td>13(86.7%)</td>
<td>2 (13.3%)</td>
</tr>
<tr>
<td>Total no. of isolates</td>
<td>43 (95.6%)</td>
<td>2* (4.4%)</td>
</tr>
<tr>
<td>No. of isolates / patient</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Range of days between isolates</td>
<td>12-18 (15)</td>
<td>12-18 (15)</td>
</tr>
<tr>
<td>No. of patient isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial isolates</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>After 15 days</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Next 15 days</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>REAP profile</td>
<td>1,2,3,4</td>
<td>2,3,5,6</td>
</tr>
</tbody>
</table>

*Only 2 (4.4%) isolates out of 45 showed RFAP difference. Their initial isolates had profile No.2,3. They respectively showed profile No, 5,6 in their sequential isolates.
DISCUSSION

The high degree of discrimination associated with typing of bacteria by DNA analyses is dependent upon an evolutionary genetic divergence arising from non lethal mutations, acquisitions, or deletions of plasmid or chromosomal DNA. If such events occur too frequently or rapidly in an isolate or strain, the applicability of DNA typing would be diminished. (Maslow et al., 1993).

The rich diversity of plasmids among MRSA suggests that there are mechanisms, perhaps involving conjugation, phage-mediated contact, transduction, transposition, and site-specific or homologous recombination that are continuously generating new plasmid forms. (Zuccharelli et al., 1990).

The rate at which these DNA reorganizations occurs has a bearing upon the usefulness of plasmid profiling to epidemiology. This application requires that the plasmid composition of pathogenic MRSA strains remain stable at least for the period of a typical infection and, hopefully, for the duration of a MRSA outbreak. (Wagenvoort et al., 2005).

In a study by Leski et al., (1998), they genetically characterize MRSA strains present in Warsaw hospital in 1992 and 1996, which were typed by phenotypic (resistogram) and genotypic (PFGE and REAP) methods. Comparison of PFGE and PEAP typing results showed PFGE was as specific as...
REAP. REAP was found to have discriminatory power similar to PFGE, but due to lack of plasmids or difficulties in plasmid isolation in 3 out of 33 studied strains, typing by REAP turned out to be lower than PFGE.

This study observed that plasmid digestion profiles of MRSA isolated repeatedly from thirteen patients over periods of up to one month remained unchanged. This suggests that plasmid rearrangements do not regularly occur within the time scale of individual infections. This results is in agreement with Hartstein et al., 1995 who found only 25 type difference among 199 sequential isolates from 39 patients collected within 200 days.

Zuccharelli et al., 1990 also reported that specific plasmid digestion profiles were persistently detected in hospitals over a period of more than 2 years.

This study demonstrated REAP difference in two isolates (4.4%) from two patients which can be explained by the occurrence of single genetic event. Hartstein et al., 1995 stated in such condition a single mutation, deletion, or insertion of DNA within a plasmid already present, occurring within the same MRSA strain or clone seemed to be the most logical explanation for these findings.

Comparison of antibiotic susceptibility tests (ASTs), plasmid profiles and REAP of MRSA and methicillin susceptible staphylococci were conducted by Tayfour et al., (2005). In their study MRSA were divided into 13 groups by ASTs, 4 groups by plasmid profiles and 5 groups by REAP. They concluded that discriminatory power of REAP > plasmid profiles > ASTs. They suggested for gathering epidemiological data, all three methods should be employed in clinical laboratories as they are practical and easily interpreted. Also automated equipment available now to accelerate plasmid extraction and make it practical for clinical laboratories.

On the other hand Krzyszton-Russian et al., (2003), typed MRSA by PFGE and REAP and found that isolates with different PFGE pattern contained plasmid with the same REAP fingerprinting.

Sabria-Leal et al. (1994) reported that during epidemic or local outbreaks several methods may be needed to best delineate the source and spread of MRSA strains. The reproducibility and discriminatory power of REAP makes it a useful tool in this context.

In a study conducted in 2002 through 2003 by Al-Thawadi et al., (2003) using REAP, randomly amplified polymeric DNA (RAPD) and PFGE for fingerprinting of bacterial isolates. They found only 20.8% of all isolates studied were of the same genotypes by all 3 methods. 2 major clusters of strains each presenting 33% of the total number of isolates were identified by
REAP. Each RAPD and PFGE however, identified one major cluster presented by 54% and 83% of the total number of isolates. All 3 methods showed clonal genetic relatedness among distant MRSA isolates. However, inter-strain comparison of fingerprint data generated from each method revealed differences in clonal representation of MRSA isolates. They illustrated the importance of using a combination of methods in typing of bacterial species.

Conclusion:

Restriction analysis of MRSA plasmids has all the attributes of a powerful epidemiological marker. The diversity and stability of plasmid profiles provide an effective means for discriminating between strains. Rapid methods for DNA extraction and data handling may make it suitable and cost effective for hospital clinical laboratories.

Recommendation:

REAP is reproducible technique and recommended for preliminary investigation of MRSA infection in hospital. It is also helpful when planning and assessing the effectiveness of interventions at endemics and outbreaks.

REFERENCES


قدرة التحليل الانزيمي للبلازيميد على التمييز والثبات في الميكروب العنقودي الكروي المقاوم للميتشللين

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تم إجراء هذه الدراسة على أربعة و خمسين عينة للميكروب العنقودي الكروي المقاوم للميتشللين. تم تجميعها من ثمانية عشر مريض بواقع ثلاث عينات متتالية من كل مريض يفصل بين كل عينة وأخرى في المتوسط. خمسة عشر يوم وذلك لاختبار قدرة التحليل الانزيمي للبلازيميد على الثبات وقدرته على التمييز بين السلالات المختلفة.

وقد وجد أن التحليل الانزيمي للبلازيميد ظل ثابتًا في ثلاثة وأربعين عينة من العينات الحاملة للبلازيميد بينما كان مختلفًا في عينتين من مريضين مختلفين. كما أثبت التحليل الانزيمي للبلازيميد قدرته على التمييز بين السلالات و تم التعرف على ستة أنماط مختلفة.

من هذه الدراسة نستخلص أن التحليل الانزيمي للبلازيميد وسيلة فعالة وقادرية في التعرف على مصدر العدوى.