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10

Pulsed Field Gel Electrophoresis in Molecular Typing and Epidemiological Detection of Methicillin Resistant *Staphylococcus aureus* (MRSA)

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1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important threat to hospitalized patients worldwide and is responsible for a wide range of human diseases, including septicemia, endocarditis, pneumonia, osteomyelitis, toxic shock syndrome, and bacteremia (Tenover & Gaynes, 2000). This species nevertheless represents a serious public health burden, particularly the clones which are resistant to methicillin and other classes of antibiotics; the emergence of penicillin-methicillin-, and recently high-level vancomycin-resistant strains emphasize the importance and urgency of such rational prescribing policy for the treatment of MRSA infections (Appelbaum, 2007; Goldstein 2007). Multiple studies have shown clonal spreads of epidemic MRSA strains within hospitals, between hospitals within a country (Breurec et al., 2011; Nübel et al., 2010), and also between countries and continents (Breurec et al., 2011; Deurenberg et al., 2009; Diekema et al. 2001). There are only a limited number of nosocomial MRSA clones spread worldwide (the Iberian [ST247-SCC*mec* I], the Brazilian [ST239-IIIA], the Hungarian [ST239-III], the New York/Japan [ST5-II], the Pediatric [ST5-VI], the Berlin [ST45-IV], EMRSA-15 [ST22-IV], and the EMRSA-16 [ST36-II] clones) (Enright et al., 2002; Oliveira et al., 2001).

Molecular typing of MRSA is used to support infection control measures. Although Pulsed-field gel electrophoresis (PFGE) is well known and considered as golden standard, for establishing clonal relationships at the local level, its detection capacity seems to make it also too discriminative for global comparisons (McDougal et al.2003; Murchan et al., 2003). Recently multilocus sequence typing (MLST) has been proven to be the most adequate method both for long-term and global epidemiologic studies and for population genetic studies. Typing methods based on sequencing of more stable housekeeping genes (MLST) allow the creation of Internet-based curate databases and inter-laboratory data exchange

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(Enright et al., 2000) The combination of these methods allows the unambiguous assignment of collections of MRSA isolates or new MRSA clones (Enright et al., 2000).

The prevalence of MRSA in Mexico differs widely from one hospital to another and according to different studies performed; an increasing frequency of MRSA (7% in 1989, 14% in 2001 and 36% in 2004) are documented by reports of routine oxacillin disk diffusion tests only (Alpuche et al., 1989; Calderón et al., 2002; Chávez, 2004). This is of great concern, because it is a common experience that once MRSA is introduced in a hospital it is difficult to eradicate it (Creamer et al., 2011; Rebmann & Aureden, 2011). However, reports from Mexico documenting the clonality of MRSA isolates are very scarce, Aires de Sousa *at al*. in 2001 (Aires de Sousa et al., 2001) reported dominant and unique MRSA clone designated the Mexican clone (I::NH::M), identified by PFGE among isolates collected in 1997, 1998 from a pediatric hospital in Mexico, which had a rather limited resistance profile. In more recent studies which involve strains collected for the period 1997 to 2003 in two Mexican hospitals, PFGE distributed the MRSA isolates into two types M (clone EMRSA-16-U.K) and C (clone New York/Japan) these two clones were distinguished by antibiogram and other molecular properties (Echaniz et al., 2006; Velazquez et al., 2004).

The aim of this study was to identify MRSA clones circulating in a tertiary care hospital in Mexico City and their prevalence in the course of time 2002-2009. For this purpose, we used a phenotypic characterization and a combination of different molecular typing methods, including PFGE, hybridization with a Tn*554* and *mecA* probes, staphylococcal cassette chromosome *mec* (SCC*mec*) and MLST.

2. Material and methods

2.1 Hospital setting

The Instituto Nacional de Cardiologia "Dr. Ignacio Chavez" (CAR) is a tertiary-care cardiology hospital located, in Mexico City with 246 beds, distributed 10 wards: surgery, adults and pediatric cardiology, neumology, nephrology, coronary unit and others. In addition the hospital has 17 external services. The microbiology laboratory receives an average of 18,000 samples annually. The hospital has 5,800 admission and 5,700 discharges per year.

2.2 Bacterial isolates

We studied a total of ninety single-patient clinical MRSA isolates, between January 2002 and December 2009. The strains were collected from several clinical sources: bronchial secretions (n=34); wound secretions (n= 25), blood (n= 16); catheter (n=3); pleural liquid (n=3); peritoneal fluid (n=1) and others (n=13). MRSA strains were collected from different wards: pediatric surgery, adult surgery, coronary unit, nephrology, surgery and cardiology. Of the 90 MRSA isolates, 24 were from children and 66 were from adults.

2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for MRSA isolates was performed using the automated method of MicroScan® (DADE-BEHRING, Sacramento, CA) for: penicillin, oxacillin,

amoxicillin, cefotaxime, cephalothin, cefazolin, imipenem, trimethoprim-sulfamethoxazole, ciprofloxacin, chloramphenicol, clindamycin, erythromycin, clarithromycin, gentamicin, rifampin, tetracycline and vancomycin, following the Clinical Laboratory Standards Institute guidelines (Clinical Laboratory Standards Institute [CLSI], 2009).

2.4 Molecular typing

The whole genomic DNA was prepared as described previously (Chung et al., 2000). After digestion with *Sma*I endonuclease, DNA was separated in a CHEF-DRII apparatus (Bio-Rad, Birmingham, U.K) (Chung et al., 2000). Strains HU25, HPV107, HDE288, BK2464, JP27 and 96/32010, representing the Brazilian, Iberian, Pediatric, New York/Japan-USA, New York/Japan-Japan and EMRSA-16-U.K clones, were included in the PFGE gels as controls. The control strains were kindly provided by Prof. Herminia de Lencastre from the Molecular Genetics laboratory Institute de Tecnología Química e Biologica da Universidad Nova de Lisboa. Criteria of Tenover were used to compare different clones (Tenover et al., 1995). Strains BK2464 and HDE288 were used as SCC*mec* controls. Hybridization of *SmaI* digests with *mecA* and Tn554 probes (de Lencastre et al., 1994), SCC*mec* typing (Oliveira & de Lencastre, 2002) and MLST (Enright et al., 2000) were performed as previously described. Briefly, MLST is based in internal fragments of seven housekeeping genes (*arcC*, *aroE*, *glpF, gmk, pta, tpi, yqiL*) for each isolates, the alleles at the seven loci defined the allelic profiles, which corresponded to a sequence type (ST). ST designations were those assigned the MLST data base (http://www.mlst.net). The SCC*mec* typing system is defined by combining the class of the *mec* gene complex with the cassette chromosome recombinase gene (*ccr*) allotypes. The polymorphism in the vicinity of the *mecA* gene detected by probe *ClaI*-digested DNAs with a *mecA* probe and transposon Tn*554* insertion patterns detecting by probing *ClaI* digestion DNAs with a specific probe (de Lencastre et al., 1994, Enright et al., 2000; Oliveira & de Lencastre, 2002).

2.5 Computer-fingerprinting analysis

The computer analysis of the banding patterns obtained by PFGE was done using the NTSYSpc software version 2.0.2.11 (Applied Biostatistics Inc.) after visual inspection. Each gel included reference strain *S. aureus* NCTC 8325 to normalize the PFGE profiles. For clusters analyses, the Dice coefficients were calculated to compute the matrix similarity and were transformed into an agglomerative cluster by the unweighted pair group method with arithmetic average (UPGMA).

3. Results

3.1 Antimicrobial susceptibility

The 90 isolates showed resistance to penicillin (100%), oxacillin (99.3%), amoxicillin (100%) , cefotaxime (100%), cephalothin (100%), cefazolin (100%), chloramphenicol (100%), imipenem (99.3%), ciprofloxacin (87.7%); eleven strains (12.2%) showed low susceptibility for clindamycin, erythromycin, clarithromycin and were susceptible to ciprofloxacin; two strains (2.2%) showed low susceptibility for oxacillin (MIC 4μg/mL) and imipenem. All strains were susceptible to rifampin, tetracycline, gentamicin, trimethoprim-sulfamethoxazole and vancomycin.

3.2 Molecular typing

3.2.1 PFGE analysis

The PFGE analysis separated the MRSA strains into three types, A (5 subtypes), B (3 subtypes) and C (6 subtypes) (Figure 1). PFGE pattern C and subtypes were predominant in this isolates n=72 (80%), Clone A, n=11 (12.2%) and B, n=7 (7.8%) were only found in the isolates of 2002, and these two clones (A and B) were totally replaced by clone C in 2004 and continue until 2009. The results produced by a computer analysis of the banding patterns show clearly the division of the three clone groups (A, B and C); interestingly, the A and B clone isolates have very similar PFGE patterns (coefficient similarity 95%). Nevertheless, the three clones A, B and C could easily be distinguished by antibiograms and other molecular properties as well (Table 1). The three clones were multiresistant, however, each one of them showed a characteristic resistance pattern; clone A was resistant to ß-lactams and showed a low susceptibility to clarithromycin, clindamycin, erythromycin and was susceptible to ciprofloxacin; while clones B and C were resistant to ß-lactams, clarithromycin, clindamycin, erytromycin and ciprofloxacin; only the strains with subtypes B1 and B2 showed low susceptibility for oxacillin and imipenem.

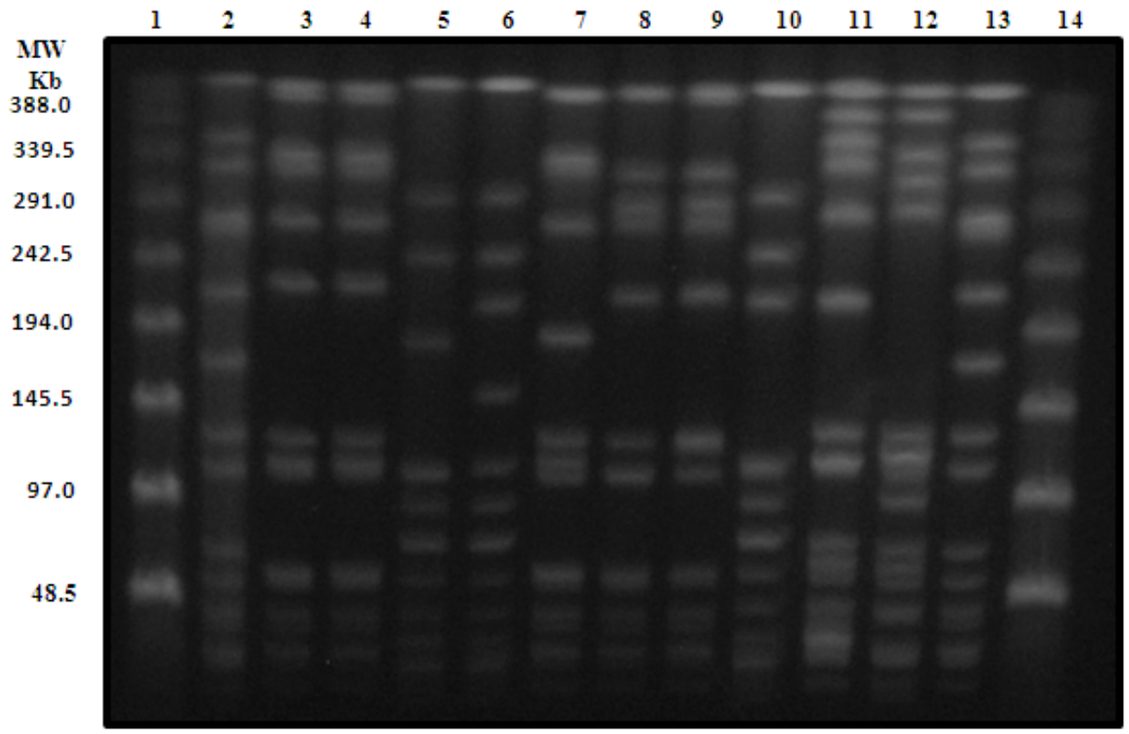

Fig. 1. Pulsed field gel electrophoresis profiles of MRSA clinical isolates from the Instituto Nacional de Cardiología "Dr. Ignacio Chávez", Mexico and representatives of international clones. Lanes: 1-14 lambda ladder used a molecular size (MW) markers; 2 and 13 reference strain NCTC8325; 3-4 (44CAR and 47CAR) pattern C; 5 (2CAR) pattern A; 6 (20CAR) pattern B; 7-12 (HDE288, BK2464, JP27, EMRSA16, HPV107 and HU25) control strains representative of Pediatric, New York/Japan-USA, New York/Japan-Japan, EMRSA16-U.K, Iberian and Brazilian clones.

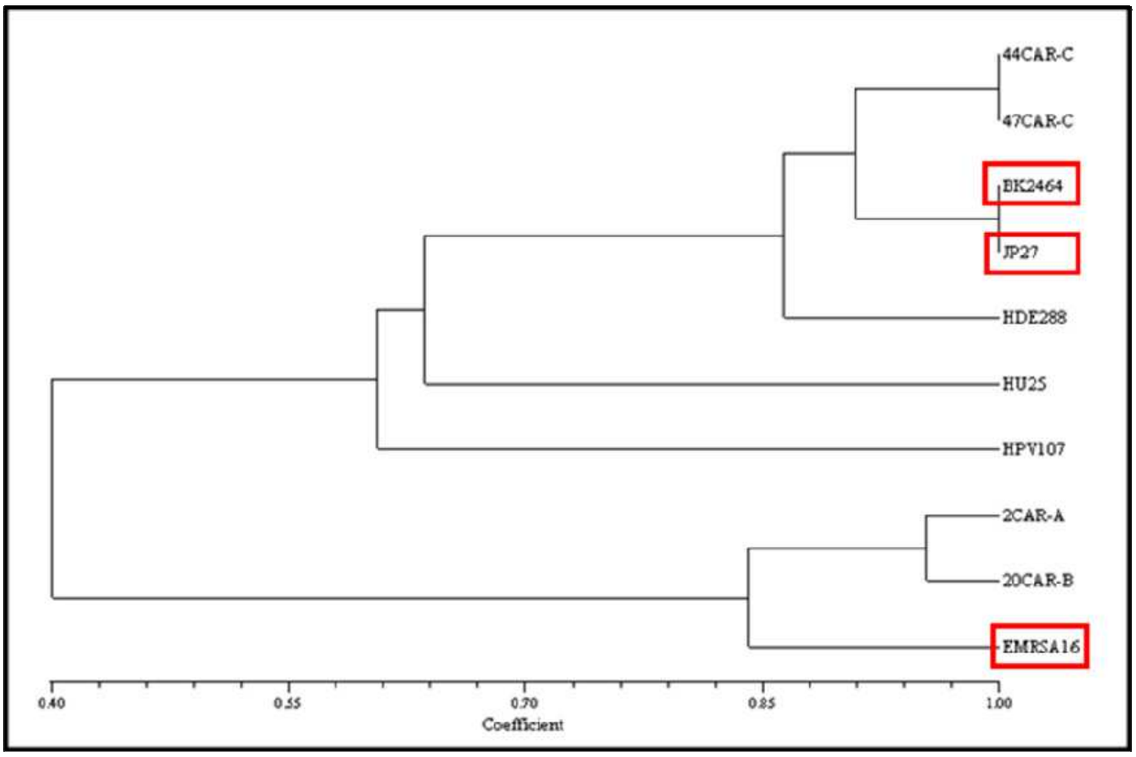

Fig. 2. Dendrogram comparing MRSA clones A, B and C from the Instituto Nacional de Cardiología "Dr. Ignacio Chávez", Mexico with different international MRSA clones: BK2464-New York/Japan-USA clone; JP27-New York/Japan-Japan clone; HDE288-Pediatric clone; HU25-Brazilian-clone; HPV107-Iberian clone; EMRSA-16-U.K. clone. For cluster analysis, Dice coefficients were calculated to compute matrix similarity a transformed into an agglomerative cluster with the unweighted pair group method with arithmetic average.

Property	Clone A	Clone B	Clone C
Antibiotype[1] (Resistance)	ß-lactams, (CLA,CD,ERY)[2]	ß-lactams, (CIP, CLA,CD, ERY)	ß-lactams, (CIP, CLA,CD, ERY)
Number of subtypes	5	3	6
SSC*mec* type[3]	IV	II	II
Hybridization bands(Kb) *SmaI-mecA*	~180	~211[4]	~211
Hybridization bands(Kb) *Sma-Tn554*	~180	~211-640	~211-640
ST	30	30[5]/36	5

[1]Antibiotic abbreviations: CLA- clarithromycin; CD – clindamycin; ERY – erythromycin GEN - gentamicin; CIP - ciprofloxacin. [2]Intermediate resistance pattern. [3]Staphylococcal cassette chromosome *mec*. [4]Except B1 and B2; [5]Sequence typing (MLST), only the patterns B1 and B2.

Table 1. Antibiotype and Genotypic characterization of the MRSA clones presented in the Instituto Nacional de Cardiología "Dr. Ignacio Chávez", Mexico (2002-2009).

3.2.2 Hybridization pattern

The hybridization patterns with *mecA* and Tn*554* probes indicated that the MRSA strains accompanying clone A carried the *mecA* gene on a *Sma*I fragment of approximately 180 Kb, while the *mecA* gene of the clones B and C were found on a fragment of approximately 211 Kb (Figure 3-A). One Tn*554* copy was identified usually on the fragment approximately 180 Kb between the isolates of clone A; while the MRSA strains accompanying clones B and C usually carried two identified Tn*554* copies between the *SmaI* fragments of approximately 211 and 640 Kb; only the strains 3CAR, (B1) and 8CAR, (B2), carried the transposon Tn*554* in a fragment of 640 Kb (Figure 3-B).

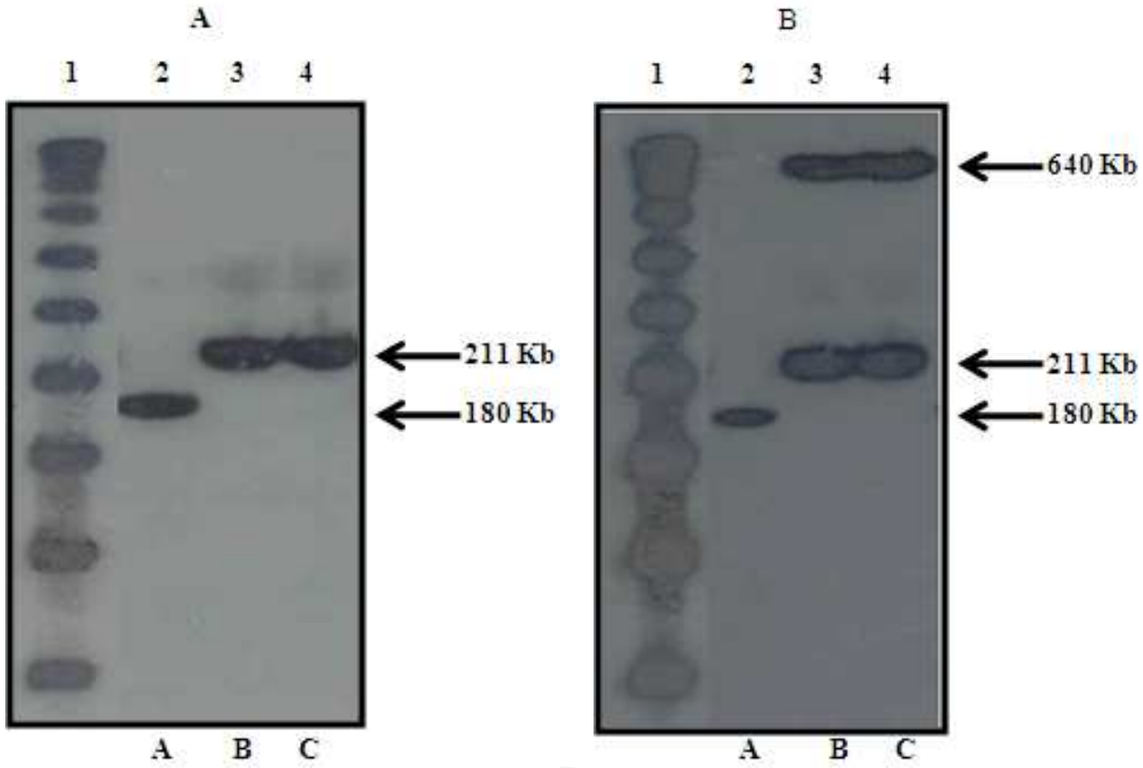

Fig. 3. (A) *SmaI-mecA* and (B) *SmaI* Tn*554* patterns identified among the clones A, B and C of the Instituto Nacional de Cardiología "Dr. Ignacio Chavéz, Mexico. Lane 1, molecular weight markers, lambda ladder; lane 2, 2CAR (pattern A); lane 3, 20CAR (pattern B) and lane 4, 44CAR (pattern C).

Two strains collected in this hospital 3CAR, (pattern B1) and 8CAR, (pattern B2) did not hybridize with the *mecA* DNA probe, interestingly both presented a low susceptibility to oxacillin and these isolates were subtypes of pattern B. The only difference was found in the *SmaI* hybridization fragment, which contains the *mecA* gene: in the two isolates, this fragment had a smaller molecular size (145 instead of 180 Kb) and did not react with the *mecA* probe, indicating a deletion of approximately 35 Kb, which must have included both the *mecA* gene and part of the *mec* element (Figure 4A and 4B). All the isolates accompanying clone A presented SCC*mec* type IV and sequence type 30 (ST30) whereas the

MRSA strains of clones B and C had SCC*mec* type II, sequences type 36 and 5 (ST36 and ST5) respectively, except B1 and B2 which did not amplify SCC*mec*, this isolates showed sequence type 30 (ST30) .

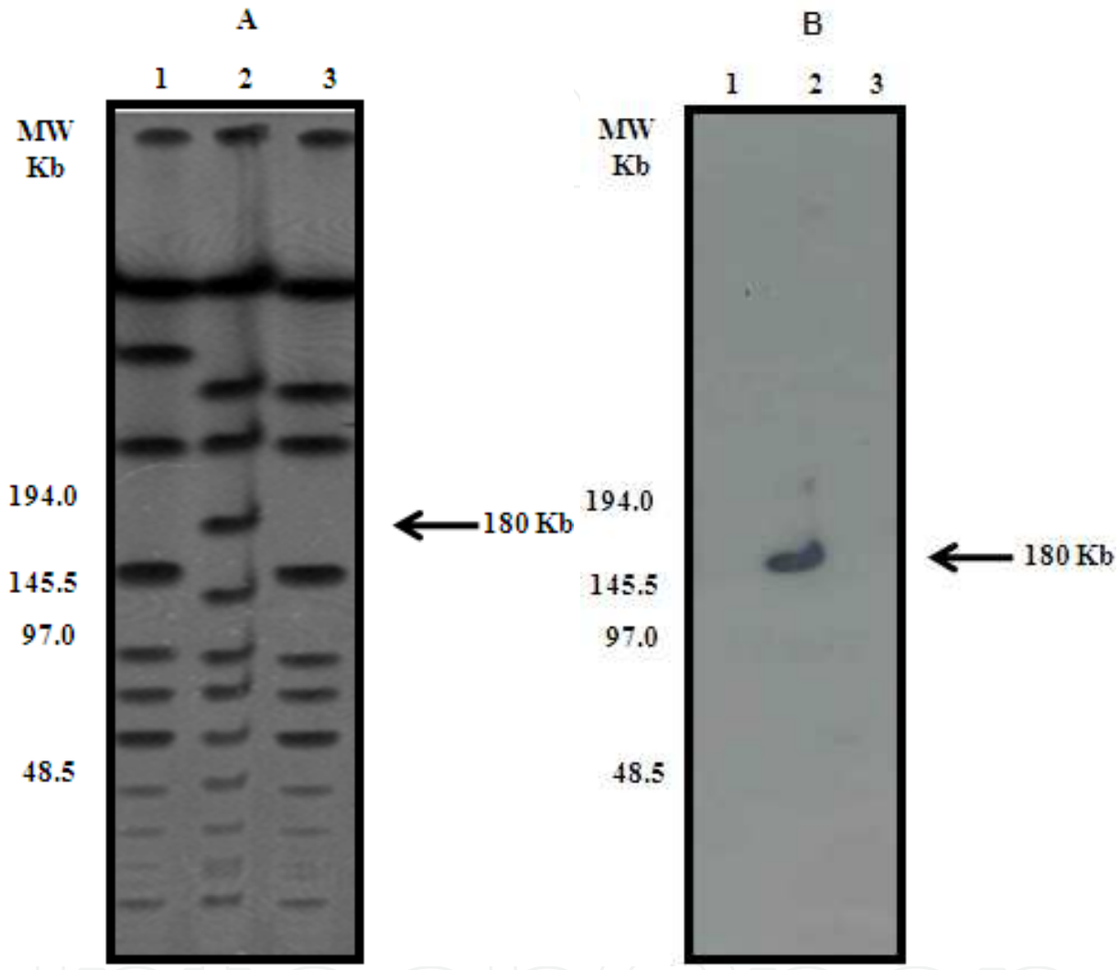

Fig. 4. (A) PFGE patterns of MRSA strains from the Instituto Nacional de Cardiología "Dr. Ignacio Chávez", Mexico. Lane 1, 3CAR, (pattern B1); lane 2, control strain and lane 3, 8CAR (pattern B2).

3.2.3 Homology pattern

One isolate belonging to each type of clones (A, B and C) were compared to strains belonging to previously characterized MRSA clones, i. e., representatives of the pediatric clone and isolates belonging to the New York-Japan clone and also to other international pandemic clones, namely, the Iberian, Brazilian and EMRSA-16 clones (Figure 1). Clone C showed a high degree of similarity to the pediatric (85.5%) and the New York-Japan (89.5%) clones. Clones A and B showed a high degree of similarity to the EMRSA-16 (80%) clone (Figure 2).

4. Discussion

The emergence of strains resistant to methicillin and other antibacterial agents has become a major concern especially in the hospital environment, because of the higher mortality due to systemic methicillin-resistant *S. aureus* infections (Cosgrove et al., 2003; Handberger et al., 2011). Seven major pandemic MRSA (the so-called Brazilian, Hungarian, Iberian, New York-Japan, pediatric, EMRSA 16 and Berlin clone (EMRSA15) have been identified as the cause for the majority of hospital-acquired *S. aureus* infections in the world (Oliveira et al., 2002), indicating that they represent successful clones in terms of their ability to cause infections, persist and spread from one geographic zone to another, including across continents.

The combination of different molecular typing methods used in the present study allowed us to register epidemiologically relevant features of MRSA populations in the Instituto Nacional de Cardiología "Dr. Ignacio Chávez" in Mexico and document the coexistence of MRSA clones of international distribution.

All 90 strains were resistant to at least eleven antibiotics (amoxicillin, cefotaxime, cephalothin, cefazolin, chloramphenicol, imipenem, clindamycin, erythromycin and clarithromycin) in addition to penicillin and oxacillin and 94.4% were resistant to ciprofloxacin as well. The phenotypes of resistance to the antimicrobial agents are shown in the Table 1.

As a response to the emergence and worldwide spread of antibiotic-resistant *S. aureus* there was an urgent need for the creation of international surveillance systems with methodologies that could help hospital infection prevention and control such organisms. MRSA causing nosocomial infections have been reported in other hospitals in Mexico showing a wide geographic spread of MRSA specific clones (Aires de Sousa et al., 2001; Echaniz et al., 2006; Velazquez et al. 2004) similar spread has been observed by other clones in USA and Europe (Da Silva, 2003; Johnson, 2011; Oliveira & de Lencastre 2002).

Interestingly, only three PFGE types were found during the period of the study, designed A, B and C. Previous studies had documented that MRSA clones may spread in and between hospitals, cities and countries and even intercontinental spread may occur (Auken et al., 2002; Nübel et al., 2010). The multiresistant clone C (New York/Japan clone) was present in more than 50% of MRSA that were recovered from a variety of infections sites and hospital wards. Previously, this clone had already been reported in two hospitals in Mexico: Hospital Civil de Guadalajara "Fray Antonio Alcalde" and Hospital de Pediatría del Centro Medico Nacional Siglo XX1-IMSS and it has been circulating in these hospitals since 1999, and 2001 respectively (Echaniz et al., 2006; Velazquez et al. 2004). The results of these studies showed that clone C (New York/Japan clone) had, sequence type 5 and SCC*mec* type II. In this study we found that pattern C was very similar (89.5%) to the multiresistant New York-Japan clone (Figure.2), which correspond to our last year´s results, proving with this the capacity of this clone to persists for long periods of time within the hospitals; as well as its capacity to spread to other hospitals (epidemic clone). whose evidence is the existence of this clone in other hospital of third level in Mexico Instituto Nacional de Cancerología (INCan). It is important to mention that the existence of clone C (New York/Japan clone) had not been present in the INCan before 2006 (Cornejo et al., 2010). All these results are of relevant importance if we consider that the first high-level VRSA (vancomycin-resistant *S. aureus*) (MIC = 1024 μg/mL vancomycin), belonged to the New York lineage (Weigel et al., 2003) and the fact that the descending MRSA strains of this clone are circulating in our

population, together with the few means of antibiotic restriction it could represent a potential short term risk for the VRSA appearance in the hospitals of our country. Clone A and B were only found in the isolates in 2002, these clones showed a high degree of similarity to the EMRSA-16 clone, this clone is one of the dominant types of MRSA found in a UK hospital (Moore & Lindsay, 2002) and was widely disseminated in Canada (Simor et al., 2002), Greece (Aires de Sousa et al., 2003) and Mexico (Aires de Sousa et al., 2001). Interestingly, both clones (A and B) are very similar (95%) (Figure 2), nevertheless, clone A showed a reduced resistance profile as clone B, and this is because of the existence of the SCC*mec* IV in these isolates, this chromosomal cassette was found in relation to isolated MRSA strains in the community (CA-MRSA) (Coombs et al., 2011). Different reports of several infections caused by CA-MRSA in Latin America (Uruguay, Rio de Janeiro, Colombia, Argentina and Mexico) have been published (Alvaréz et al., 2006; Ma et al., 2005; Reyes et al., 2009; Ribeiro et al., 2005; Velazquez et al., 2011). All pattern of PFGE of the clones A, B and C showed subtypes. Probably the PFGE subtypes indicate the continued evolutionary divergence of these clones during its massive geographic expansion.

Relative genetic instability of the *mecA* element was observed in two strains and this was associated with an apparent deletion of the *mec* element, these isolates were very similar to profile B (B1 and B2) and presented a low susceptibility to oxacillin. In the literature there are reports of *S. aureus* strains with low-level methicillin resistance (MIC 2-4 µg/mL) which are not associated to the presence of *mecA* gene, Tomasz et al. reported one class of borderline methicillin-resistant strains having PBP1 and PBP2 with altered methicillin-binding affinities and overproduction of PBP4 (Tomasz et al., 1989). Another class of low-susceptibility has been reported and was attributed to overproduction of penicillinase (McDougal & Thornsberry, 1986). Hackbarth et al. studied the nucleotide sequence of the PBP2 gene and identified a point mutation near the penicillin-binding motive of transpeptidase (Hackbarth et al., 1995). An MRSA clinical strain with significant methicillin resistance (MIC 64µg/mL) despite absence of *mec A* was reported (Yoshida et al., 2003).

5. Conclusion

The combination of molecular typing methods (PFGE, *mecA*, Tn*554* probes, SCC*mec*, and MLST) with epidemiologic and clinical information allows the detection of MRSA clusters and outbreaks and therefore provides a rationale for appropriate infection control intervention. Our study emphasizes the need of national and international collaborations to monitor the spread of current epidemic strains as well as the emergence of new ones in our country. The mechanisms of spread in different areas are poorly understood and further studies are necessary to understand the dynamics involved in the predominance of unique MRSA clones.

6. Acknowledgment

We thank PhD. Lilia Chihu for style review of the paper

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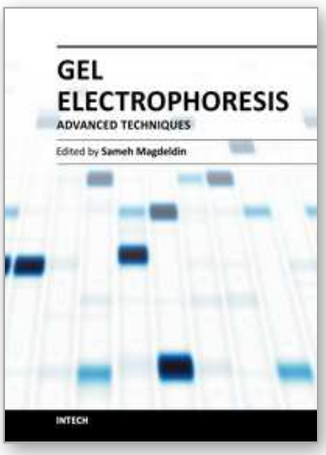

Gel Electrophoresis - Advanced Techniques

Edited by Dr. Sameh Magdeldin

ISBN 978-953-51-0457-5

Hard cover, 500 pages

Publisher InTech

Published online 04, April, 2012

Published in print edition April, 2012

As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Velazquez-Meza Maria Elena, Vázquez-Larios Rosario, Hernández Dueñas Ana Maria and Rivera Martínez Eduardo (2012). Pulsed Field Gel Electrophoresis in Molecular Typing and Epidemiological Detection of Methicillin Resistant Staphylococcus aureus (MRSA), Gel Electrophoresis - Advanced Techniques, Dr. Sameh Magdeldin (Ed.), ISBN: 978-953-51-0457-5, InTech, Available from: http://www.intechopen.com/books/gel-electrophoresis-advanced-techniques/pulsed-field-gel-electrophoresis-in-molecular-typing-and-epidemiological-detection-of-methicillin-re

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