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Molecular study of methicillin-resistant *Staphylococcus aureus* isolates at a neonatal high-risk unit in Merida, Venezuela

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Background:

Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multiresistant microorganisms which holds first place in the world as a nosocomial pathogen. Special attention has therefore been directed to specific nosocomial surveillance systems and strict infection control measures for this microorganism in which the microbiological laboratory plays an important role by applying phenotypic and genotypic methods that permit establishing their epidemiological relationship especially in hospital outbreaks. In the present study the general objective was to study MRSA strains isolated from neonates with nosocomial infections and from healthcare personnel working in the Neonatal High Risk Unit (NHRU) of the Andes University Hospital Autonomous Institute (AUHAI) in Mérida, Venezuela.

Material/Methods:

Forty-three *S. aureus* isolates were analyzed by phenotypic and genotypic methods.

Results:

In these strains, antibiotics resistant to oxacillin, gentamicin, erythromycin, and tobramycin predominated (50%). The greater percentage of MRSA strains isolated from health personnel as well as two neonates were described as pulse types Ia and Ib, belonging to phage group II, containing type IV *SCCmecA* and resistant to macrolides and aminoglycosides and sensitive to clindamycin and trimethoprim-sulfamethoxazole.

Conclusions:

This is the first reported case of *SCCmecA* type IV MRSA found in the NHRU of the AUHAI.

key words:

MRSA • phagetype • antibiotic • PFGE • PCR • *SCCmecA* • ICUn • CA-MRSA

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BACKGROUND

Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus* resistant to vancomycin are important microorganisms associated with infections of hospital origin. In the epidemiology of staphylococcal infections at the hospital level, it is known that patients found in high-risk areas, such as intensive care units, are the most susceptible to acquiring infection by this type of microorganism [1–3]. Healthcare personnel as well as patients colonized with or infected by MRSA represent the main reservoir for transmission of MRSA in hospital environments. Diverse studies show that such personnel play a predominant role in the cross-transmission of MRSA [4–6]. MRSA infections that occur in otherwise healthy persons who have not been recently hospitalized or had a medical procedure are known as community-associated infections. These infections are usually skin infections, such as abscesses, boils, and other pus-filled lesions [7]. MRSA may also lead to severe pneumonia even in immunocompetent people without previous hospitalization or healthcare contact [8].

Strains that are methicillin resistant are resistant to all β -lactam agents, including cephalosporin and carbapenems. Vancomycin, linezolid, and daptomycin are among the drugs that are used for the treatment of severe healthcare-associated MRSA infections [9]. Participation of the microbiology laboratory in the surveillance and control of nosocomial infection is critical in the event of outbreaks, given that one of its functions is to determine the source of infection when comparing strains involved to establish its identity and type [2,10]. Of the methods of characterization used for the study of MRSA, phagetype and antibiotype are indicated as phenotypic markers as well as the analysis of DNA (plasmidic and chromosomal) [11–13].

Because MRSA is an important nosocomial pathogen at the Neonatal High Risk Unit (NHRU) of the Andes University Hospital Autonomous Institute (AUHAI) [5], Mérida, Venezuela, the general objective of the present study was to characterize by phenotypic and genotypic methods MRSA strains isolated from neonates with nosocomial infection as well as healthcare personnel working in the mentioned area.

MATERIAL AND METHODS

Forty-three strains of *S. aureus* isolated in different time periods from different carriers (healthcare personnel) and neonates diagnosed with nosocomial infection hospitalized in the NHRU of the AUHAI were evaluated. They were stored at -20°C until the time of testing. Of the 43 strains of *S. aureus*, eight were isolated from neonates with nosocomial infection (1998–1999) and 35 were from healthcare personnel working in the NHRU (3 in 1998, 17 in 2002, and 17 in 2003). *S. aureus* ATCC 25923 was used as a control strain. Bacterial identification was carried out according to procedures established by Koneman et al. [14].

Determining antimicrobial susceptibility

Disk-diffusion method

The susceptibility of each *S. aureus* strain was determined for the following antimicrobial agents: penicillin (10 U), oxacillin (1 μg), gentamicin (10 μg), tobramycin (30 μg),

erythromycin (15 μg), chloramphenicol (30 μg), clindamycin (2 μg), rifampicin (5 μg), tetracycline (30 μg), vancomycin (30 μg), and trimethoprim/sulfamethoxazole (1.25/23.75 μg), all from OXOID laboratory, using a qualitative disk-diffusion method, the Kirby-Bauer (KB) technique, according to the procedure described by the Clinical Laboratory Standard Institute (CLSI) [15].

Agar dilution method

The minimum inhibitory concentrations (MICs) were determined by the agar dilution method according to the procedure described by the CLSI [15] for the resistant *S. aureus* strains by the disk-diffusion method to gentamicin, erythromycin, tobramycin, and oxacillin. The drugs were provided by PROULA Laboratories (erythromycin) and Valmorca Laboratories (tobramycin, gentamicin, and oxacillin). A strain was considered resistant to erythromycin, gentamicin, and tobramycin when they had an inhibitor concentration ≥ 8 $\mu\text{g}/\text{ml}$ and to oxacillin ≥ 4 $\mu\text{g}/\text{ml}$.

Detection of heteroresistance to oxacillin

Heteroresistance to oxacillin was determined by culture of the *S. aureus* strains on Mueller Hinton agar plates containing 4% NaCl and 6 $\mu\text{g}/\text{ml}$ of oxacillin. The test was considered positive upon observing microbial growth after 24 hours of incubation at 35°C [15].

Phage typing

All the MRSA strains were subjected to typing using the international set of phages of *S. aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) provided by the Central Public Health Laboratory of London according to the method previously described by Parker [16].

MecA gen detection:

The polymerase chain reaction (PCR) was used in an attempt to amplify an internal fragment of the *mecA* gene of all the strains of MRSA according to the previously described method of Geha et al. [17]. The subunit 16S RNAr was amplified with the primers X (5'-GGA ATT CAA A [T/G, I: 1] G AAT TGA CGG GGG C) and primer Y (5'-CGG GAT CCC AGG CCC GGG AAC CTA TTC AC). The *mecA* gene was amplified with the *mecA1* (5' – GTA GGA ATG ACT GAA CGT CCG ATA A) and *mecA2* (5'CCA ATT CCA CAT TGT TTC GGT CTA A) primers. The PCR parameters were 94°C for 45 s, 50°C for 45 s, and 72°C for 60 s for 30 cycles, ending with an extension time of 2 minutes. The amplified product was visualized in an 1.2% agarose gel using ethidium bromide and a transilluminator.

Staphylococcal cassette chromosomal *mecA* (SCC *mecA*) type detection

Detection of the SCC *mecA* type using multiple PCR was carried out on the MRSA strains that had the *mecA* gene according to the previously described procedure [18].

Pulsed-Field Gel Electrophoresis (PFGE) typification

PFGE according to the previously described procedure [19] was carried out on the *S. aureus* strains within phage group

Table 1. Antimicrobial resistance patterns and phage groups of methicillin-resistant *Staphylococcus aureus* strains of hospital origin in Mérida-Venezuela.

Phage group/antimicrobial resistance patterns	Strains (n)
Phage group I	
Ox Gm E Tob	3
Ox Gm E ^I Tob	1
Phage group II	
Ox Tob	1
Ox E	1
Ox E Tob	1
Ox E ^I	1
Ox Gm E	2
Ox Gm E Tob	7
Ox Gm E ^I Tob	1
Phage group III	
Ox Gm E	1
Ox Gm E Tob	2
Ox Gm E ^I Tob	2
Phage group I-III	
Ox Gm Tob	1
Ox Gm E ^I Tob	2
Subtotal	3
Inverse Phage type	
Ox Gm E Tob	3
Ox Gm E ^I Tob	1
Total	30

Ox – oxacillin; Gm – gentamycin; E – erythromycin; Tob – tobramycin.

II. The SmaI enzyme was used for DNA chromosomal digestion. PFGE was carried out with CHEF-DRII electrophoresis equipment in 1% agarose gel for 22 h at 6 V/cm and 12°C. The gels were dyed with ethidium bromide and visualized with a UV transilluminator. Banding patterns were inspected manually and the criteria of Tenover et al. [20] were used to determine the strains' relatedness.

RESULTS

Of the 43 strains of *S. aureus* studied, 76.7%, 74.4%, 74.0%, and 72.1% showed resistance to gentamicin, erythromycin, tobramycin, and oxacillin, respectively, using the agar dilution method. All the strains were sensitive to the rest of the test antibiotics. Of the MRSA strains, 75% showed heteroresistance to oxacillin. The resistance antibody type to oxacillin, gentamicin, erythromycin, and tobramycin (50%) was pre-



Figure 1. *MecA* gene Detection on methicillin resistant *Staphylococcus aureus* of hospital origin, Mérida-Venezuela. 1. » (100 pb) Phage; 2. Mix; 3. N 071; 4. N 088; 5. N 428; 6. N 495 (1988); 7. N 509; 8. N 526 B; 9. N 458.1 (1999); 10. LAN 13.1; 16. RAN 13.2; 12. RAN 14.1; 13. LAN 18.4; 14. LAN 20.1 (2002); 15. RAN 1.1; 16. RAN 3.1; 17. RAN 17.1; 18. RAN 20.4; 19. LAN 22.3 (2003); 20. gene *mecA* control positive; 21. LAN 27.1; 22. LAN 28.2; 23. RAN 31.3; 24. LAN 32.2; 25. H34,3; 26. H36.1; 27. H 37.1 (2003); 28. HPC 1.3; 29. HPC 1.4; 30. HPC 11.4 (1998). LAN – Left Anterior Nare, RAN – Right Anterior Nare, H – Hand, N – Neonates, HPC – Health Personal Carrier.

dominant in the *S. aureus* strains studied (Table 1). On the other hand, conventional phage typing showed that 47% of the MRSA strains belonged to phage group II (Table 1). The MRSA strains were not typed by the international phage sets specified by MRSA. Using PCR, the presence of the *mecA* gene was determined in 86.6% of the MRSA strains (26/30) (Figure 1). All the MRSA strains belonged to phage group II and had SCC *mecA* type IV (Figure 2, Table 2), which in the majority came from nasal samples as well as on the hands of carriers working in the NHRU. The PFGE technique revealed three pulse types in the MRSA strains located in the phage group II (type Ia: 72%, type Ib: 14%, and type II: 14%) (Figure 3, Table 2).

DISCUSSION

MRSA is a Gram-positive coccus that has had great impact on the clinical-epidemiological environment of public health on a global level due to its high frequency as an etiological infectious agent in different ecosystems (veterinary, the general community, and especially hospital environments) [21,22]. In the NHRU of the AUHAI, multiresistant MRSA strains causing infection in neonates as well as in health personnel working there [5] have been isolated. Continuing the epidemiological observation, in the present study the phenotypic and genotypic characteristics of some of the MRSA strains recovered from neonates between 1998–1999 as well as strains isolated from healthcare personnel, i.e. nasal and hand carriers who worked in the mentioned study area from 2002 and 2003, and which showed similar phenotypic resistance were researched.

S. aureus is a microorganism of interest not only for showing resistance to methicillin, which implies resistance to all the β -lactamic antibiotics, but also for the resistance it has also shown to other groups of antimicrobial agents, such as aminoglycosides, macrolides, lincosamines, and rifampicins, and even reduced susceptibility to glycopeptides, which have caused a state of epidemiological alert at the international level. Similar results were obtained in the present study in

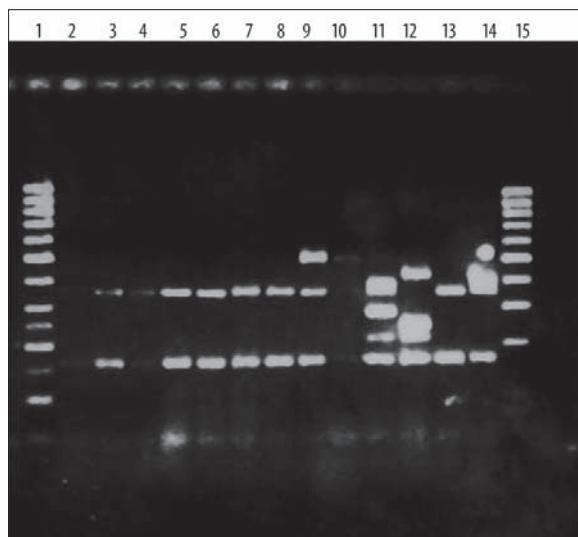


Figure 2. Staphylococcal cassette chromosome (SCC) *mecA* gene type detection on methicillin resistant *Staphylococcus aureus* strains of hospital origin. Mèrida-Venezuela. 1: λ Phage (50 pb); 2: Mix; 3 N.088 (1998); 4. HPC 11.4 (1988); 5. RAN 13.1 (2002); 6. LAN 17.1 (2003); 7. RAN 20.4 (2003); 8. RAN 22.3 (2003); 9. Control SCC *mecA* type I; 10: Control SCC *mecA* type IA; 11. Control SCC *mecA* type II; 12. Control SCC *mecA* type III A; 13. Control SCC *mecA* type IV; 14. Control SCC *MecA* type IV A; 15. λ Phage (100 pb). LAN – Left Anterior Nare, RAN – Right Anterior Nare, N – Neonates, HPC – Health Personal Carrier.

which an elevated percentage of MRSA strains were resistant to more than three antimicrobial agents, mainly aminoglycosides and macrolides. However, in contrast to hospital-acquired MRSA (HA-MRSA) strains reported in other studies [23–26], all the strains studied here were sensitive to clindamycin and trimethoprim-sulfamethoxazole. *Staphylococcus* spp. resistance to macrolide, lincosamide, and streptogramin B (MLS_R) is often mediated by small non-conjugative plasmids carrying the *ermC* determinant. This type of resistance can be constitutive (cMLS_R) or inducible (iMLS_R) [27]. When an organism shows iMLS_R-type resistance to erythromycin, the clindamycin should be administered with caution [28]. In addition to the significant increase in the prevalence of MRSA at the international level, the development of multiple resistance to aminoglycosides and other antimicrobial agents has been observed. More than 60% of the MRSA strains studied in the present investigation were resistant to gentamicin and tobramycin, results similar to those reported by Ardić et al. [29], who found the genes encoding aminoglycoside-modifying enzymes together with the *mecA* gene in 72% of the MRSA strains evaluated.

When resistance was first described in 1961, methicillin was used to test and treat infections caused by *S. aureus*. However, oxacillin, which is in the same class of drugs as methicillin, was chosen as the agent of choice for testing staphylococci in the early 1990s. In addition to the fact that methicillin is no longer commercially available in many countries, oxacillin maintains its activity during storage better than methicillin and is more likely to detect heteroresistant strains [30]. Resistance to oxacillin is expressed by two phenotypes: ho-

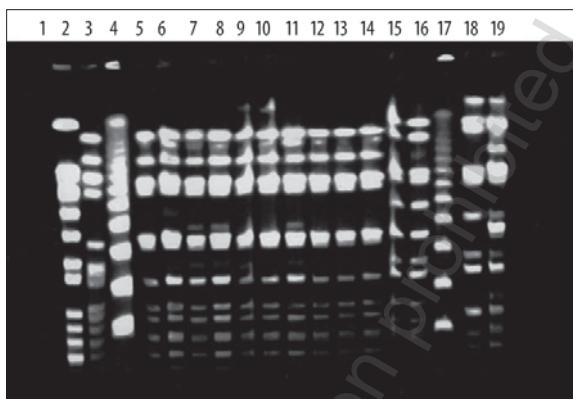


Figure 3. Pulsed Field Electrophoresis (PFGE) Typing of methicillin resistant *Staphylococcus aureus* phagegroup II of hospital origin, Mèrida-Venezuela. 1. (-); 2. Control strain 50; 3. Control strain methicillin sensitive *Staphylococcus aureus*; 4. Phage »; 5. RAN 1.1 (2003); 6. RAN 3.1 (2003); 7. RAN 17.1 (2003); 8. LAN 20.4 (2003); 9. LAN 22.3 (2003); 10. LAN 27.1 (2003); 11. RAN 31.3 (2003); 12. M 34.3 (2003); 13. M 36.1 (2003); 14. N 071 (1998); 15. LAN 13.1 (2002); 16. LAN 20.1 (2002); 17. Phage »; 18. LAN 13.1 (2002); 19. HPC 11.4 (1998). LAN – Left Anterior Nare, RAN – Right Anterior Nare, H – Hand, N – Neonates, HPC – Health Personal Carrier.

mogeneous and heterogeneous (heteroresistance), which may coexist within a culture of staphylococci. All cells in a culture many carry genetic information for resistance, but only a small number may express that resistance *in vitro* to penicillinase-stable penicillins such as oxacillin [30]. The latter caused greater problems from the microbiological point of view given that its detection in the laboratory is difficult to determine and, as such, at any given time a false sensitivity of *S. aureus* to oxacillin could be reported, causing an inadequate therapeutic handling of the patient. As a result, these strains can be disseminated in the hospital environment in patients as well as in healthcare personnel. The Z-252 phage-type MRSA clone has been observed in 17 hospitals in different places in Holland. In other countries of Europe, unexpected epidemic strains of MRSA have been reported with heteroresistant phenotypic expression [31]. In the present study a significant percentage of MRSA strains showing heteroresistance to oxacillin were found.

The *mecA* gene leads to the synthesis of an altered penicillin-binding protein, called PBP2a [32,30]. PCR is the gold standard for the detection of the *mecA* gene in MRSA strains [33,34] and this gene was found in the majority of the strains studied in the present study. Not all microbiology laboratories have the infrastructure available to carry out PCR; however, they could have several methodologies to detect the *mecA* gene indirectly, such as the direct detection of PBP2a for agglutination with latex particles as well as the method of diffusion with a cephoxitin disc (30 μ g/ml), as recommended by the CLSI [15] for the indirect detection of PBP2a, which can be used in routine work as the discs are more economical and easily executed tools for rapidly detecting the *mecA* gene in MRSA strains.

S. aureus phage groups I, III, and I-III only come from hospital environments, in contrast to phage group II strains

Table 2. Characteristics of methicillin-resistant *Staphylococcus aureus* strains of hospital origin; Mérida, Venezuela.

Strain	Origin	Period	Phage group or inverse phage type	Resistance pattern	mec A gene Type**	Pulse type PFGE***
N071*	N	1998	II	Ox Gm E Tob	IV	la
N599.1	N	1999	II	Ox Gm E Tob	IV	lb
HPC11.4*	HPC	1998	3A 3C 6 47 83A	Ox Gm E Tob	IV	II
LAN 13.1*	HPC	2002	3A 3C 6 47 83A 85	Ox Gm E ^l Tob	IV	II
LAN 20.1*	HPC	2002	II	Ox Gm E Tob	IV	lb
RAN 1.1*	HPC	Jul-03	II	Ox Tob	IV	la
RAN 3.1*	HPC	Jul-03	II	Ox Gm E	IV	la
RAN 17.1*	HPC	Jul-03	II	Ox Gm E Tob	IV	la
RAN 20.4*	HPC	Jul-03	II	Ox Gm E ^l Tob	IV	la
LAN 22.3	HPC	Jul-03	II	Ox Gm E Tob	IV	la
LAN 28.2	HPC	Jul-03	II	Ox Gm E Tob	IV	la
RAN 31,3*	HPC	Jul-03	II	Ox E ^l	IV	la
H 34.3*	HPC	Jul-03	II	Ox Gm E Tob	IV	la
H 36.1*	HPC	Jul-03	II	Ox Gm E Tob	IV	la

LAN – Left Anterior Nare; RAN – Right Anterior Nare; N – Neonates; H – Hand; HPC – Health Personnel Carrier.

* Oxacillin heteroresistant; ** Polymerase Chain Reaction (PCR); *** Pulsed-Field Gel Electrophoresis (PFGE).

Ox – oxacillin; Gm – gentamycin; E – eritromycin; Tob – tobramycin.

that are only found in the community [35–37]. This differs from the results obtained in the present study, in which 47% (14/30) of the *S. aureus* strains were found in phage group II. This could be explained by theories that have been postulated on the exchange of *S. aureus* strains between the different hospital and community environments [38,39]. On the other hand, the MRSA strains in the present study were unique to the study area and did not come from the dissemination of a clone from European countries. Typing by bacteriophages, based on susceptibility to the lytic activity of bacteriophages, has been widely used for over 50 years. With the advent of molecular methods, especially PFGE, this technique has been displaced. However, it is still an internationally recognized method which, despite its low discriminatory power, can be useful if it is used in combination with other genotypic methods in the identification of the principle routes of dissemination of a certain strain [37].

The study of polymorphisms of SCC *mecA* has been used as a typification method in studies concerning the molecular epidemiology of MRSA. Various types of SCC *mecA*, types I, II, and III, have been found in MRSA strains of hospital origin. On the other hand, types IV and V SCC *mecA* have been detected in community-acquired MRSA strains (CA-MRSA). However, Trindade et al. [40] showed the presence of these two last types in hospital environments, just as we did in the present study, where all the MRSA strains belonging to phage group II showed the presence of type IV SCC *mecA*.

SCC *mecA* type IV is rarely found in MRSA strains associated with personnel in charge of healthcare. In contrast, this

type is predominant in CA-MRSA, the majority of which shows resistance to methicillin, is typically heterogeneous, and can be susceptible to certain β -lactamics such as the carbapenems. Some strains of CA-MRSA are highly virulent and produce Panton-Valentine leukocidin [41]. In contrast, HA-MRSA SCC *mecA* types I, II, or III have multiple determinants of antimicrobial resistance and are typically multiresistant [42]. In the present study the majority of the MRSA strains isolated from healthcare personnel and two isolated from neonates with nosocomial infection were found to have characteristics of MRSA strains acquired in the community: they were grouped in phage group II, they had SCC *mecA* type IV, and were still multiresistant, especially to macrolides and aminoglycosides, and conserved susceptibility to clindamycin. In relation to the latter aspect, Popovich et al. [43] proposed a rule based on the antibiotic type to predict the genotype which can be applied for epidemiological purposes to describe the tendency of CA-MRSA over time. According to the various phenotypic rules tested by Popovich et al. [43], fluoroquinolone susceptibility or a combination of fluoroquinolone or clindamycin susceptibilities were better in predicting the community genotype. De Sousa and De Lecantre [44] found similarities between isolated sporadic CA-MRSA and HA-MRSA strains, increasing the possibility that at least some of the SARM strains described as being acquired in the community can originate from a hospital. Jemili-Ben Jomaa et al. [24] also reported the existence of CA-MRSA strains with SCC *mecA* types I and III and multiresistance, which can be a consequence of the easy transfer between the hospital environment and the community. Reports also exist that introduce MRSA clones

from the community in the hospital [45,25], just as it occurred in the present study. The current tendency seems to be that MRSA strains that emerge from the community will be disseminated in the community and can potentially disseminate into the hospital [23]. On the other hand, PFGE revealed that pulse types Ia and Ib with CA-MRSA characteristics isolated from neonates with nosocomial infection in 1998 remained in the unit during the study carried out in 2003. HA-MRSA has been the main cause of infection in neonatal populations. Recently, reports of CA-MRSA have moved their threat to maternity and children wards. The majority of CA-MRSA strains in newborns are acquired through skin-to-skin transmission with colonized adults, which can result in bacteremia and systemic infection [3].

To control and prevent MRSA in the NHRU, the following activities are recommended: hand hygiene, isolation of the neonates infected, observation of neonatal cultures, decolonization, environmental cultures, and molecular analysis when researching an outbreak [1].

CONCLUSIONS

This study is the first national report of CA-MRSA clones (*SCC mecA* type IV) present in a neonatal high-risk unit where healthcare personnel, made up of medical doctors and nurses, are the main reservoir of this microorganism.

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