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Original Article

Molecular Characterization of Staphylococcus aureus Isolates Obtained from Hemodialyzed Patients at the Hospital de Clínicas of Paraguay: A pilot study

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Abstract

Background: Patients undergoing hemodialysis are susceptible to the nasal carriage of Staphylococcus aureus, increasing the risk of developing infections associated with higher morbidity and mortality. The objective of this study was to describe the frequency of S. aureus carriage in hemodialysis patients and to perform molecular analysis of isolates by applying multiple-locus variable analysis. **Methods:** We conducted a descriptive cross sectional study with non-probabilistic sampling that included 28 hemodialysis patients attending the Nephrology Department of Hospital de Clínicas in Asunción, Paraguay. We obtained clinical data from medical records and interviews with patients. Nasal swabs were collected and analyzed by microbiological and molecular methods. **Results:** The frequency of S. aureus carriage was 50% (14/28), 93% of which (13/14) were methicillin resistant, 57% (6/14) were gentamicin resistant and 36% (5/14) were resistant to more than 4 antibiotic classes. S. aureus carriers showed higher frequency of rhinitis (p=0.02 odds ratio [OR]=6.6 (1.2-34.4)). Seven methicillin-resistant S. aureus isolates had been analyzed by multiple-locus variable analysis, two of them showed identical pattern bands. **Conclusion:** We found a high frequency of methicillin-resistant Staphylococcus aureus colonization and the presence of two isolates with identical profile in the multiple-locus variable analysis indicating the possibility of transmission between patients.

Keywords: Staphylococcus aureus, Hemodialysis, Antibiotic resistance, bacterial typing (Source: MeSH-NLM).

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Introduction

S. aureus is a common pathogen causing bloodstream infections in the hospital environment. The possibility of nasopharyngeal colonization increases the risk of endogenous infections, and is linked to the 80% of cases of invasive S. aureus infections.[1] This microorganism was frequently reported as a pathogen in patients undergoing dialysis and kidney transplantation.[2] These individuals have risk factors for colonization and infection with multidrug-resistant S. aureus because they are exposed to frequent and prolonged use of antimicrobials.[3] Furthermore, the need to use invasive devices such as catheters for venous access are associated with a high risk of bloodstream infections.[4,5] In Paraguay the data about this pathogen in hemodialysis patients is scarce and is limited to local studies that have not been published. Other risk factors for methicillin resistant S. aureus (MRSA) carriage are age . 75 years, prolonged hospitalization, history of repeated administration of antibiotics, type of vascular access, the frequency of hospitalization, immunosuppressive therapy, the use of heparin in the middle of treatment, iron overload, lack of hygiene, comorbidities and proximity to other people carrying S. aureus .[6,7]

Despite the great technological advances, the mortality rates in hemodialyzed (HD) patients remain unsatisfactorily high. Along with cardiovascular disease, infections are the leading causes of morbidity, hospitalization and mortality in this population. The annual mortality rate for sepsis is 100 to 300 times higher in patients with end stage renal disease than in the general population, and there is evidence of the association between nasal carriage of MRSA and poor clinical outcome in HD outpatients.[6-8]

Molecular typing has been used to perform epidemiological studies at the global level. In a specific environment over a short period of time, microorganism typing techniques are used to study nosocomial outbreaks, local transmission, and the relationship between carriage and infection in patients.[9]

The multiple-locus variable number of tandem repeat analysis (MLVA) method can be used for the analysis of genetic variability of S. aureus isolates because it has a high discriminatory power for the characterization of bacterial isolates, and it is based on the variation of 7 different loci. The analysis generates multiple PCR products that differs in size for each allele and produces a pattern of 7 bands like a code bar. Isolates that are genetically distant present differences in their profile of bands and those that are identical share the same pattern of bands.[3,10] The objective of this pilot study was to describe the frequency of S. aureus carriage in hemodialyzed patients attending the nephrology department of the Hospital de Clínicas, and perform the clustering analysis of isolates by the MLVA molecular technique.

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Table 1. Clinical outcomes when comparing the interventions.

	Sensible % (n)	Resistance % (n)
Oxacillin	7%(1)	93%(13)
Erythromycin	21%(3)	79%(11)
Ciprofloxacin	29%(4)	71%(10)
Clindamycin	36%(5)	64%(9)
Gentamicin	43%(6)	57%(8)

Patients and Methods

Study Design

A descriptive cross-sectional pilot study with a non-probabilistic sampling of consecutive cases was performed on October 2013, including 28 patients undergoing hemodialysis in the Nephrology Department of Hospital de Clínicas from Paraguay. The study was approved by the Facultad de Ciencias Médicas Ethics Committee of the Universidad Nacional de Asunción. Patients received information about the study and those who agreed to participate voluntarily signed an informed consent form. For the data collection process, we interviewed patients and revised the medical records. Two patients with missing data concerning this study were excluded. The data collected included the patient's age, sex, years undergoing hemodialysis, white blood cell count, differential count of lymphocytes, neutrophils and eosinophils expressed as number of cells/mm3, day and turn of hemodialysis, use of antibiotics over the last six months, the use of an invasive device, and history of rhinitis.

Collection of samples

We collected the nasal swab of dialysis patients and preserved them in Stuart medium for transportation to the microbiology laboratory. The samples were cultured on blood agar, chocolate agar, mannitol agar and incubated at 37°C for 24 hours. The identification of S. aureus (STAPH-PLUS Pastorex, USA) included the following tests: Gram staining and biochemical test of catalase, DNase, and the agglutination test.

Susceptibility

We have implemented the disk diffusion Kirby-Bauer method according to the Clinical and Laboratory Standards Institute (CLSI) in order to identify the antimicrobial susceptibility. We evaluated the susceptibility to oxacillin, ciprofloxacin, erythromycin, ampicillin-sulbactam, clindamycin, and gentamicin as well. The susceptibility to oxacillin was tested through the use of a cefoxitin disk considered as a breakpoint for resistance if the growing inhibition zone of diameter ,21mm. Susceptibility to vancomycin was not evaluated due to lack of access to methods to determine the minimum inhibitory concentration. Intermediate susceptibility results were considered resistant.

Molecular Methods

We confirmed isolates as S. aureus molecularly through the amplification of a specific 16S rRNA gene, using the protocol and oligonucleotides described by Manfredi et al. 201011. We have extracted DNA from isolated colonies on blood agar by using the commercial kit Wizard Genomic (Promega, USA) following the manufacturer instructions. DNA quantification was performed using the UV spectrophotometer Biowave DNA (Cambridge, UK). The analysis of the genetic variability of S. aureus isolates was carried out by the MLVA technique. This technique involves amplifying seven loci using oligonucleotides and was previously described by Sabat and collaborators.[12] The strain ATCC®29213, negative mecA and producing weak β -lactamase was included as a control in each MLVA assay. We separated the PCR products on 7.5% polyacrylamide gels and performed silver staining following the protocol described by Sambrook et al.[13] We captured digital images with the Kodak Digital Science team CD120 system (Kodak, NYC, United States). We used TreeCon 1.3b software (Ghent University, Gante, Belgium) for the analysis of the band patterns generated and the design of dendrograms.

Statistical issues

We have compared continuous variables such as: patients age, years undergoing hemodialysis, white blood cell count, differential count of lymphocytes, neutrophils, and eosinophils (expressed as number of cells/mm3) between patients with MRSA carriage versus patients without MRSA carriage through the Student's t-test; and dichotomous variables as well such as: patients sex, day and turn of hemodialysis, use of antibiotics over the last six months, the use of an invasive device, and history of rhinitis were analyzed using the chi-square test. Statistical significance was defined as a p value of $p<0.05$ using SPSS 15.0 software (IBM, NYC, United States) for statistical analysis.

Table 2. Resistance profile of Staphylococcus aureus isolates.

	Percentage (n)
More than 1 antibiotic classes	93%(13)
More than 2 antibiotic classes	86%(12)
More than 3 antibiotic classes	50% (7)
More than 4 antibiotic classes	36% (5)

Results

Among the 28 HD patients from the Nephrology Department of Hospital de Clínicas who were part of this study, 50% (14/28) of them were carriers of S. aureus. Antibiotic susceptibility testing identified 92.9% (13/14) of MRSA carriage. Isolates were also resistant to other antibiotics, especially erythromycin and ampicillin-sulbactam, both with 78.6% (11/14). 42.9 % (6/14) of the isolates were sensible to Gentamicin (Table 1). Isolates that showed resistance to more than 4 families of antibiotics were considered multiresistant and included 35.7% (5/14) of MRSA isolates (Table 2).

The analysis of the demographic and clinical data of the hemodialyzed patients classified as: S. aureus carriers and non-carriers showed that only the differences registered in rhinitis OR=6.6 (95% CI: 1.2 – 34.4, p=0.022) were statistically significant (p<0.05) (Table 3).

All the isolates identified as S.aureus by biochemical methods (n=14) were confirmed by the detection of 16S rRNA gene by PCR amplification, with a 100% agreement between phenotypic and genotypic methods. PCR amplification of the 7 loci included in the MLVA method were optimal for cluster analysis in 7 of the 14 MRSA isolates in the study and it gave us the chance to determine the genetic variability (Table 4). The bioinformatic analysis of the band profiles obtained in these isolates generated a dendrogram which is showed in Figure 1. This graphic display has the form of a tree, in which the distance between braches indicates the genetic difference. It can be observed that isolates identified as CR-4 and CR-5 show the same band profile and are clustered together in one branch at the same distance, whereas isolates CR-10 and CR-12, and CR-9 and CR-14 as well, show similar but not identical band profiles and are clustered in groups with branches relatively close to each other (Figure 1).

Table 3. Demographics of Hemodialyzed Patients

	Staphylococcus aureus Carriers (n=14)	Staphylococcus aureus Non Carriers (n=14)
Age (Mean±SD)	41±14	44±18
Dialysis Turn		
Evening	43% (6)	71% (10)
Afternoon	50% (7)	29% (4)
Days of dialysis		
Monday, Wednesday, Friday	43% (6)	71% (10)
Tuesday, Thursday, Saturday	57% (8)	29% (4)
Time of dialysis treatment in years (Mean±SD)	6.1±5.5	5.1±4.6
Use of Arteriovenous fistula	79% (11)	64% (9)
History of different Vascular Access (Mean±SD)	2±2	3±2
Antibiotic treatment in the last 6 months	36%(5)	29% (4)
Rhinitis[a]	64% (9)	21% (3)
Laboratory data (Mean±SD)		
Leucocytes	7510±3767	6012±1531
Neutrophils	5443±3609	3788±1143
Lymphocytes	1509±333	1602±572
Eosinophils	263±302	243±333
Albumin (g/dL)	3.7±0.24	4.01±0.69
Hemoglobin (g/dL)	9.74±1.71	9.08±2.03

[a] Difference was statistically significant.

Discussion

The results showed an alarming frequency of Staphylococcus aureus carriage in hemodialyzed patients (50%), considering that carrying this organism is an important cause of morbidity and mortality in patients who receive hemodialysis. This study portrayed a high prevalence of nasal S. aureus carriage compared with others studies[14,15] that showed between 5-13% of S. aureus carriage.[14,15] However, two studies demonstrated similar carriage rates of S. aureus in hemodialysis patients including Verhoeven et al (58%)[16] and Price et al (49%).[17] The risk of infections in nasal carriers of this microorganism is real and well defined.[18] It is important to point out that the small number of patients included in this study is a limitation and that the results cannot be extrapolated to other hemodialysis services. However, the data regarding this topic is the first published in Paraguay and further research or systematic studies could be done throughout the country in the future.

There are several studies on MRSA carriage, which reported variable data. One meta-analysis by Zacharioudakis et al.19 found 6% of carriage; the frequency in this study was much higher (46.4%). MRSA carriers had an increased risk of mortality in all diseases. This is attributable to the characteristics of the patients that are carriers of MRSA, who exhibit an impaired immune response, and as a consequence lead to an increased

Table 4. Phenotypical and Genotypical Characteristics of Isolates Typed by MLVA.

Patient	Sex	Age	HD Turn	Isolate	O	E	A	L	G	C	MLVA Cluster
4	M	53	Afternoon	CR-4	R	R	R	R	R	R	A
5	M	38	Afternoon	CR-5	R	R	R	R	R	R	A
9	F	57	Evening	CR-9	R	R	R	I	R	I	E
10	M	58	Afternoon	CR-10	I	I	I	R	S	S	B
12	M	37	Afternoon	CR-12	S	S	S	S	S	S	C
14	F	32	Evening	CR-14	R	S	I	S	I	I	F
17	M	20	Evening	CR-17	R	R	R	R	R	R	D

MLVA: Multiple Locus Variable Number of Tandem Repeat Analysis; HD: Hemodialysis; CR: Sample code; M=Male; F=Female; R=Resistant; I=Intermediate; S=Sensitive; O=Methicillin; E=Erythromycin; A=Ampicillin-sulbactam; L=Clindamycin; G=Gentamicin; C=Ciprofloxacin.

risk of infections.[8] Risk factors predispose not only for MRSA carriage, but also for multidrug-resistant gram negative bacilli (GNB) and vancomycin-resistant enterococci (VRE).[20] There is the possibility of vancomycin resistance in MRSA, through the transmission of the vanA gene, as occurred in neighboring countries.[21] In accordance with the high level of MRSA carriage, 32% of the isolates in the study were resistant to all antibiotics used in the Kirby-Bauer test, especially erythromycin (79%). Gentamicin was the antibiotic which had the most effective action in-vitro (43% sensitivity), but the resistance rates were high as well. This study showed similar rates of resistance compared to other countries within the region.[22] The high rates of resistance to antibiotics by MRSA display the capability of the pathogen to carry more resistance genes than MSSA.

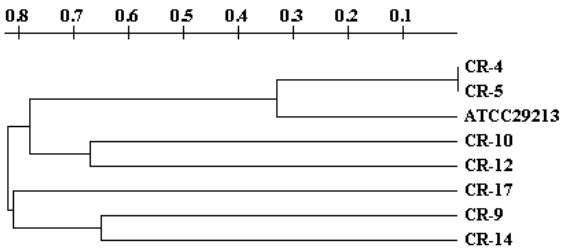
It is important to mention that we did not test the susceptibility to vancomycin in this study; this would be relevant for the epidemiological surveillance and behavior of strains in the hospital environment. In our country, VISA (vancomycin-intermediate S. aureus) or hVISA (heterogeneous vancomycin-intermediate S. aureus) have not been reported yet. This issue could be associated with the restricted access to an automated method to test vancomycin resistance by the minimum inhibitory concentration (MIC) method that is not available in all microbiology laboratories.

By using the MLVA technique, we identified 2 isolates that showed identical band profiles. These isolates also shared the same antimicrobial spectrum (CR-4, CR-5), showing the possibility that they correspond to the same clone. This should be analyzed by the combination of other molecular methods such as field gel electrophoresis (PFGE), multilocus sequencing typing and spa typing.

We discarded the possibility of epidemiological outbreaks due to the low number of isolates having identical MLVA profiles, but we have not excluded any risk of transmission between patients in the service. Internal transmission taking place in the hemodialysis service is a risk, considering that multi-resistant bacteria are circulating. The major goal of MLVA is to determine if isolates are epidemiologically linked and followed over a relatively short period of time to see if they are related or unrelated. In this study, MLVA was helpful for discarding outbreaks in a hospital setting. MLVA is useful, fast, easy to perform, and particularly cheaper to the PFGE method already used in PCR-based assays.[23,24] In respect to other variables that were analyzed in patients, the highest frequency of rhinitis may be related to the fundamental role of eosinophils in the production of itching and predisposition to allergic reactions.[25] Additionally, the nasal carriage of S. aureus produces an immunomodulatory effect that could contribute to airway inflammation and allergic response in patients with allergic rhinitis.[26] In the case of patients undergoing hemodialysis, presenting symptoms or signs may be overlapped by uremic pruritus.[27] Study limitations were the low number of samples included as it was difficult to extrapolate results to all patients undergoing hemodialysis. Nevertheless, as a pilot study this generated the first set of data on the characterization of S. aureus from hemodialysis patients in Paraguay. As a perspective, we are interested in extending the study to other centers throughout the country.

The spread of S. aureus can be controlled through reinforcement of appropriate use of antibiotics, hand washing and laboratory surveillance for S. aureus, particularly in the nosocomial wards, in order to identify sources of outbreaks. The application of MLVA could help us to elucidate if isolates are epidemiologically linked, useful information for confirming or discarding outbreaks in the hospital environment.[28,29]

Figure 1. Genetic Relationship Observed by MLVA among Staphylococcus aureus Isolates



Legend: Dendrogram obtained with the software TreeCon v1.3b. Seven offourteen isolates were processed by the MLVA technique for cluster analysis including a control ATCC®29213). The dendrogram shows the relationship between isolates, the abscissa numbers are indicative of the percentage of genetic variability. For example, between isolates CR-4 and CR-5 there is no variability, but between CR-4 and CR-17 there is 80% genetic variability.

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Author Contributions
Conception and design the work/idea: RASI, CRCV, GAVR, JP, RMGF. Collect data/obtaining results: RASI, CRCV, GAVR. Analysis and interpretation of data: RASI, FR, RMGF. Write the manuscript: RASI. Critical revision of the manuscript: FR, E. Approval of the final version: RASI, FR, CRCV, GAVR, JP, RMGF. Contribution of patients or study material: CRCV, GAVR, JP. Administrative or technical advice: FR, JP, RMGF..

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