Identificação de Anticorpos Anti-PBP2a em Pacientes Colonizados e Infectados por *Staphylococcus aureus* Resistente a Meticilina

Identification of Anti-PBP2a Antibodies in Patients Colonized and Infected by Methicillin Resistant *Staphylococcus aureus* (MRSA)

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Resumo

Infecções causadas por *Staphylococcus aureus* resistente a Meticilina (MRSA) são especialmente problemáticas devido às dificuldades de tratamento e alta taxa de mortalidade associada. O principal determinante do amplo espectro de resistência aos beta-lactâmicos em cepas de MRSA é a proteína de ligação à penicilina 2a (PBP2a). A PBP2a está localizada na superfície exterior, que seriam acessíveis ao sistema imune. Não está totalmente caracterizado se o hospedeiro pode produzir anticorpos anti- PBP2a durante infecções por MRSA ou se estes anticorpos seriam protetores. O objetivo deste estudo foi investigar a presença de anticorpos anti- PBP2a em um grupo de 60 pacientes colonizados ou infectados por MRSA. Também foi investigado se estes anticorpos poderiam reduzir o crescimento bacteriano em um ensaio *in vitro*. Através da técnica de ELISA, os resultados mostraram que cerca de 70% das amostras apresentaram anticorpos anti- PBP2a (colonizados: 68,6% versus doentes infectados: 72 %), confirmado pelo teste Western Blot. Também foi avaliado o efeito bactericida do soro contendo anticorpos anti-PBP2a e soros controle. Uma redução do crescimento bacteriano foi observada em soros de anticorpos anti - PBP2a, em comparação com os controles. Os resultados indicam que pacientes infectados ou colonizados por MRSA produzem anticorpos contra PBP2a e que estes anticorpos pode conferir proteção contra a MRSA.

Palavras-chave: Infecção. Anticorpos. Infecção Hospitalar.

Abstract

Infections caused by methicillin-resistant Staphylococcus aureus (MRSA) are especially troublesome because of treatment difficulties and high mortality rate. The major determinant of the broad-spectrum beta-lactam resistance in MRSA strains is the penicillin-binding protein 2a (PBP2a). Since PBP2a is located on the outer surface, it would be accessible to the immune system. It is not fully characterized whether the host can produce anti-PBP2a antibodies during MRSA infections or whether these antibodies would be protective. The aim of this study was to investigate the presence of anti-PBP2a antibodies in a group of 60 patients colonized or infected by MRSA. It was also investigated whether these antibodies can reduce bacterial growth in an in vitro assay. Via the ELISA technique, the results showed that approximately 70% of the samples had anti-PBP2a antibodies (colonized: 68.6% versus infected patients: 72%), confirmed by the Western blotting assay. The bactericidal effect of serum containing anti-PBP2a antibodies and control sera were also evaluated. A reduction in bacterial growth was observed in the anti-PBP2a antibody sera as compared to the controls. The results indicated that MRSA-colonized or-infected patients produce antibodies against PBP2a, which can confer protection against MRSA.

Keywords: *Infection. Antibodies. Cross Infection.*

1 Introduction

Methicillin resistant *Staphylococcus aureus* - MRSA is of great concern in many hospitals worldwide. MRSA infections are particularly troublesome due to the difficulties inherent in treatment and the high mortality rate[1]. Several years ago, an alternative treatment of MRSA infections using glycopeptides became available. However, the emergence of vancomycin intermediate - VISA and vancomycin resistant *S. aureus* - VRSA strains have led many clinicians to fear that the efficacy of vancomycin may soon become compromised in treating emerging MRSA-resistant strains[2].

Consequently, new treatment regimens are urgently required to control the infections caused by this pathogen. As such, active and passive immunotherapy seems to be a promising option. Staphylococcal surface proteins exposed during *in vivo* contact between the pathogen and its hosts are considered potential targets for immunotherapy, and several vaccine candidates have been tested focusing on these proteins[3-5]. However, to evaluate the immune response generated by these targets, host versus pathogen interactions should be analyzed. In this context, two distinct clinical situations should be considered: colonization and infection, keeping in mind the important differences between them such as host immune status, bacterial load, and length of exposure to the pathogen.

Nasal carriage is a characteristic feature of Staphylococci. Although multiple sites can be colonized in human beings,

the anterior nares are the most frequent carriage site for *S. aureus*[6]. Historically, individuals have been classified regarding *S. aureus* carriage into three groups: persistent carriers (~ 20% of individuals), intermittent carriers (~30%), and non-carriers (~50%)[7]. However, the prevalence of MRSA nasal carriage is low, varying anywhere from 1.6 to 3% in the community as a whole versus the hospital setting, respectively[8,9]. Nasal colonization occurs among healthy individuals, including health-care workers and hospitalized patients[10]. Moreover, it has been shown that nasal carriers of *S. aureus* have an increased risk of being infected with this pathogen[6]. Nosocomial infections caused by MRSA, commonly referred to as opportunistic infections, most often occur in intensive care units due to the immunocompromised state of the patients[11].

Located outside of the bacterial membrane[12], PBP2a is a transpeptidase enzyme with low affinity to all beta-lactam antibiotics[13]. This enzyme has been shown to elicit protective antibodies in two different DNA vaccine studies using a murine model[14,15]. Despite these promising results, there is no information available to date about the capacity of MRSA-colonized or infected patients to produce anti-PBP2a antibodies.

The present study identifies and compares the location of anti-PBP2a antibodies in groups of MRSA-colonized or -infected patients. In addition, an investigation was launched into the ability of anti-PBP2a antibodies to reduce bacterial growth in an *in vitro* assay.

2 Material and Methods

2.1 Human serum samples

Serum samples were collected from colonized and infected patients hospitalized at the Hospital do Amparo in Rio de Janeiro, R.J., Brazil, and the Hospital de Pronto Socorro in Porto Alegre, Rio Grande do Sul, Brazil. Colonized patients were defined as those from whom MRSA from anterior nares was isolated despite the absence of clinical signs of infection. Nasal samples were obtained with sterile cotton swabs, which were placed in Stuart transport medium, and transported to and processed in the microbiology laboratory within 4 hours. Cotton swabs were plated in ChromAgar MRSA® and incubated at 37 ºC for 24 hours. The presence of pink colonies was indicative of MRSA strains. Antimicrobial susceptibility testing was performed according to Clinical Laboratory Standards Institute (CLSI) guidelines. Sera from MRSA-negative and colonized by methicillin sensitive *Staphylococcus aureus* (MSSA, PBP2a negative) patients were collected as negative controls. Serum samples were stored at – 20 ºC for subsequent analysis.

2.2 Bacterial strain

A strain of the Epidemic Brazilian Clone MRSA[16] was donated by Dr Agnes Figueiredo. These bacteria were employed in the serum bactericidal assay, as described in Section 2.5.

2.3 Enzyme-linked immunosorbent assay (ELISA)

NUNC-maxisorp (NUNC) were coated with purified recombinant PBP2a fragment (5 mg/mL) diluted in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. Plates were washed three times with PBS containing Tween 20 (0.5%), and blocked for 2 hours at 37°C with blocking buffer (5% fat-free milk solution in PBS). After three washing cycles, sera (1:100) were added to the blocking buffer and incubated for 2 hours at 37 °C. Bounded antibodies were detected using peroxidase-goat anti-human IGs (GAM) (BioManguinhos Brazil) diluted at 1:5000 in the blocking buffer and incubated for 90 minutes at 37 °C. After three washing cycles, the substrate (TMB-peroxidase BioManguinhos Brazil) was added and the plates were incubated for 15 minutes at room temperature in the dark. H_2SO_4 0.1 N was added to stop the reaction. Absorbance was read at 450 nm in a Microplate Reader Model 680 (BioRad - USA). Pools of normal serum taken from patients noncolonized and non infected by MRSA or *S.aureus* and from patients colonized and infected by MSSA were used as negative controls.

2.4 Western blot

The recombinant purified PBP2a fragment was resolved in 15% SDS-polyacrilamide gel using standard techniques[17]. Protein was electrophoretically transferred to a nitrocellulose membrane (TransBlot, BioRad, USA). The membrane was cut into strips and each strip was blocked for 2 hours in 5% fat-free solution in a PBS solution at room temperature and under gentle agitation. The strips were washed and put into contact with diluted (1:250) sera and incubated for 2 hours at room temperature under gentle agitation. Bounded antibodies were detected with a phosphatase alkaline conjugate anti-human IGs (GAM) secondary antibody (Sigma, USA, diluted 1:15.000). Blots were developed with phosphatase alkaline reagent (Western Blue, Promega, USA). Sera from MSSA colonized patients and non-infected patients were used as negative controls.

2.5 Serum bactericidal assay

A serum bactericidal assay was performed, as previously described[18]. Bactericidal assays were performed by measuring the change in bacterial titer over time in the presence of 90% inactive (heated at 56 °C for 30 min) sera from pooled anti-PBP2a positive (colonized/infected MRSA sera), anti-PBP2a negative (colonized/infected MSSA sera), and a negative control (serum samples from non-colonized/non-infected patients by *S.aureus*). An inoculum of approximately 1 x 10^5 CFU of MRSA was utilized, and

titers were measured at 0, 1, 2, and 3 hours.

2.6 Statistical analysis

The Mann-Whitney test was used to determine the significant differences between the colonized and infected groups in the ELISA test and in their growth rates in the serum bactericidal assay. The differences were considered positive at $p<0.05$.

2.7 Ethics Committee evaluation

This study was approved by the Ethics Committee of Instituto de Pesquisa Clínica Evandro Chagas – FIOCRUZ-Brazil (Ethic Committe # 0009.0.009.000-08).

3 Results and Discussion

3.1 Serum samples

Sixty serum samples were selected from 35 colonized and 25 infected patients, respectively. Samples were obtained from patients between 20 and 81 years of age. Most of both the colonized and infected patients were hospitalized in the ICU (intensive care unit) for more than seven days. Approximately 68% of these patients were administered antibiotic treatment (data not shown). Bacteria were recovered from anterior nares in all colonized patients, and from blood, sputum, secretions, and other sites (such as wounds and catheters) of infected patients. Sera of colonized and infected patients were selected within the first 2 weeks after MRSA was identified. Patient clinical features are described in Table 1.

Table 1: Characteristics of the colonized and infected patients selected for this study

	Colonized patients	Infected patients
Age	26-83 (45.5*)	20-77 (43.8*)
Ward	ICU -33 (94.3%) Clinical ward – 02 (5.7%)	ICU – 12 (48%) Clinical ward – 03 (12%) Burn ward – 05 (20%) Traumatology – 03 (12%) Cardiology – 02 (8%)
MRSA recovery site	Anterior nares - 35 (100%)	Blood – 7 (28%) Sputum – 10 (40%) Secretions – 5 (20%) Others – 3 (12%)
Total	35	25

(*) mean.

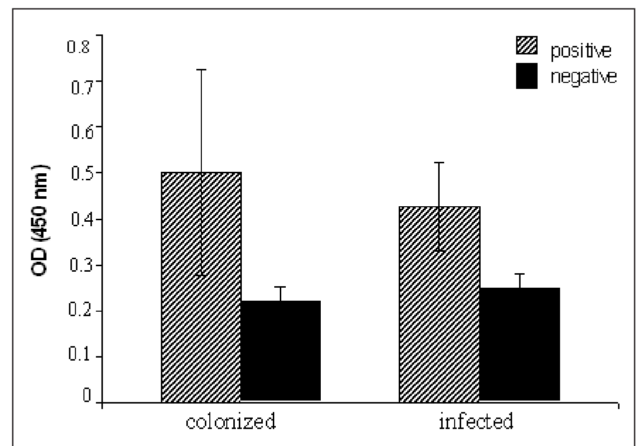
Source: Research Data.

3.2 ELISA

Samples from the infected and colonized patients were analysed in duplicate. Sera from MSSA colonized and non-colonized/non-infected patients showed a 0.3 optical density (OD), which was adopted as the cut-off value in determining whether samples were positive or negative to anti-PBP2a antibodies. According to these criteria, 70% of the overall samples were considered positive (colonized: 68.6%; infected: 72%). The ODs of analysed sera ranged from 0.125 to 1.275; and the mean was 0.502 and 0.418 for colonized and infected sera, respectively.

Statistical analysis indicated that there was no significant difference between the colonized and infected samples (p 0.5655). However, despite the small difference between the mean OD of colonized and infected sera, the highest individual OD scores were found among the colonized patients. ELISA results are shown in Figure 1.

Figure 1: Optical densities of colonized and infected MRSA patients sera measured via ELISA



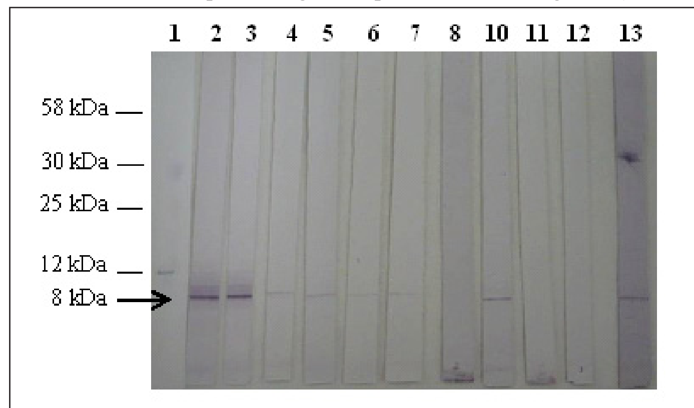
Source: Research Data.

As shown in Figure 1, the diluted samples (1:100) were analyzed in duplicate. The figure represents the average of positive and negative results of each colonized and infected MRSA sera with the standard deviation. Colonized patient serum corresponds to 35 samples (24 positive and 9 negative results); and infected patient serum corresponds to 25 samples (18 positive and 7 negative results). No significant difference was found between the colonized and infected samples (Mann-Whitney test, $p>0.05$).

3.3 Western blot

Since the human serum samples did not have a positive control, a Western blot employing the purified recombinant PBP2 a fragment[19] was used to confirm ELISA results. All samples considered negative by ELISA were also negative via Western blot analysis. Three samples tested positively by ELISA (two from colonized and one from an infected patient, respectively) and negative by Western blot assay were further considered as negative. According to ELISA, the ODs of all these negative samples were close to 0.3. Figure 2 shows examples from some sera analyzed by Western blot.

Figure 2: Western blot probed with serum taken from MRSA-colonized and MRSA-infected patients against a purified PBP2a fragment (~ 8 kDa)



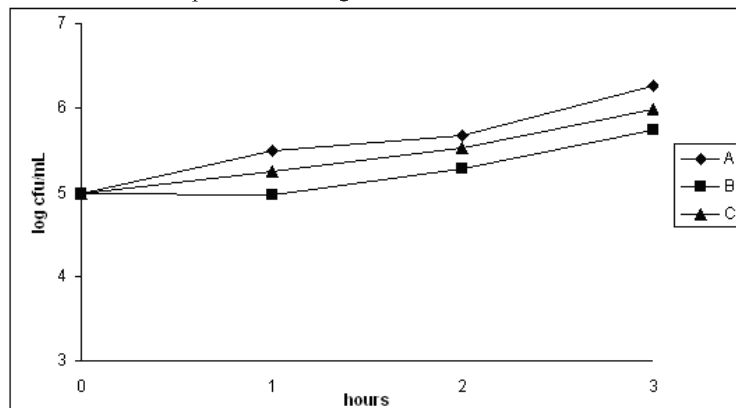
Lane 1: Protein marker (Kaleidoskope 161-0324 BioRad - USA) with the molecular mass of proteins indicated on the left. Lanes 2 and 3: Positive colonized-patient sera showing the highest ODs by ELISA. Lanes 4, 5, 6 and 7: Positive infected-patient sera. Lane 8: Negative sera (by ELISA and Western blot). Lanes 10 and 13: Positive colonized-patient sera. Lanes 11 and 12: Negative controls: Serum from MSSA-colonized patient serum (Lane 11), and serum from a non-colonized/non-infected patient (Lane 12).

Source: Research Data.

3.4 Serum bactericidal assay

To investigate whether serum containing anti-PBP2a antibodies can interfere in *in vitro* bacterial growth, a MRSA strain was grown in the presence of selected patient sera. Sera containing anti-PBP2a antibodies were selected from the three samples presenting the highest ODs via ELISA. Samples from infected or colonized MSSA patients and from non-colonized/non-infected *S.aureus* patients were used as controls. These control sera showed lower than 0.3 ODs in ELISA and were negative in the Western blot assay, confirming their negativity in the presence of anti-PBP2a antibodies. Fewer bacteria were recovered in the sera containing anti-PBP2a antibodies when compared to the controls in all (at 1, 2, and 3 hours) trials. The mean reduction in bacterial growth among non-colonized/non-infected sera and samples containing anti-PBP2a antibodies was 66.5 %; whereas, among samples of patients colonized by MSSA and those containing anti-PBP2a antibodies, it was 46.7 %. In this study, two independent assays presented similar results (Figure 3). Two independent assays were performed and each point represents the mean of these assays. Although the survival of bacteria in the presence of sera containing anti-PBP2a antibodies is lower than the survival rate in both controls, these reductions were not considered significant (Mann-Whitney test: $p > 0.05$).

Figure 3. Effect of 90% pooled human sera from (**A**) non-colonized/infected by *S.aureus* patients, (**B**) MRSA colonized/infected patients and (**C**) MSSA colonized/infected patients on the growth of the MRSA strain



Source: Research Data.

Staphylococci are isolated from skin and mucous membranes of humans and animals, colonizing the normal microbiota. These organisms may become pathogenic in the presence of predisposing factors such as after the use of

probes, immunosuppression, antibiotic therapy, *Diabetes mellitus*, and others[20]. Prior colonization has been considered a risk factor for development of infection and, in the case of multidrug-resistant strains such as MRSA, infection severity increases due to difficulties in administering treatment[21]. The present study evaluated the location of anti-PBP2a antibodies in the sera of MRSA-colonized and MRSA-infected patients. As a rule, healthy colonized patients were considered immunocompetent, and it was assumed that a large percentage of hospitalized MRSA-infected patients could be immunosupressed. Thus, a higher level of anti-PBP2a antibodies was expected to be found in colonized than in MRSA-infected patients. However, practically the same titers of antibodies anti-PBP2a were found in both groups (68.6 and 72% were considered positive, respectively). One possible explanation is that all the patients were hospitalized and most of the colonized patients were in intensive care. Some of these colonized patients had most probably been exposed to the same risk factors as the infected patients, i.e., previous antibiotic therapy and a long ICU stay. The low antibody levels elicited in both groups may be due to the period in which serum sampling took place. Breeding was only possible up to the second week after MRSA identification, because several patients had been either discharged or transferred to other hospitals.

The presence of antibodies against staphylococci in human sera has been reported by Dryla and colleagues and Lorenz and colleagues[22,23]. In another study, Kolata and colleagues studied serum samples collected from 2023 patients at hospital admission and found that only 12 of these patients developed an *S.aureus* bacteremia[24]. In a large clinical study performed in The Netherlands, carriers had a better outcome of *S.aureus* bacteremia than non-carriers[25]. These studies have shown that *S.aureus* antibodies can be elicited by carriers and can be a role in the prevention of *S.aureus* infections.

In our study, serum containing anti-PBP2a antibodies was able to generate a reduction in bacterial growth. The number of recovered bacteria grown in serum containing anti-PBP2a antibodies was 1.75 to 2.31 times lower than the number found in MSSA colonized-patient (absence of anti-PBP2a antibodies) sera at all times in both experiments. When comparing with the other controls (bacteria grown in the pooled serum from non-colonized/non-infected *S.aureus* patients), this reduction ranged from 2.52 to 3.39 times lower.

The presence of specific anti-PBP2a antibodies in colonized and infected patients should be evaluated differently than the presence of antibodies against staphylococci, as previously reported. In these studies, elicited antibodies had probably been generated long before the study began, as can be surmised due to the previous patients contact with commensal staphylococci. Because MRSA is not a commensal bacterium, it can be assumed that this specific immune response against PBP2a was generated at a recent date, probably immediately after hospitalization. However, it is very difficult to accurately estimate the elicited protection level *in vivo*. The present study demonstrated that these anti-PBP2a antibodies in the sera of infected and colonized patients were able to decisively reduced bacterial growth in an *in vitro* serum bactericidal assay.

It was also shown that while sera containing anti-PBP2a antibodies were able to reduce bacterial growth, these results must be carefully analyzed. Once both bacteria (MRSA and MSSA) probably generate antibodies against staphylococcal surface proteins, it could be conjectured that the difference in the number of recovered bacteria is due to the anti-PBP2a antibodies present in the serum of patients colonized or infected by MRSA. However, it is not certain that both samples had the same or similar anti-staphylococcus antibody levels.

Despite the complexity of this subject and the above-mentioned limitations of our study, the results clearly indicate that MRSA infected and colonized patients are able to produce anti-PBP2a antibodies, which, in turn, most likely confer some level of protection against MRSA, as previously shown in studies involving DNA immunization in mice[14,15]. Thus, our results importantly suggest that immunotherapy strategy employing high amounts of purified and specific anti-PBP2a antibodies can be a promising approach in the treatment of infections caused by MRSA. Since it has been demonstrated that antibodies anti-PBP2a can confer protection, the development of vaccine aiming at nasal decolonization may be a promising strategy to prevent MRSA nasal carriage among health care workers and patients.

4 Conclusion

Our results indicated that: (i) MRSA colonized or infected patients produce antibodies against PBP2a, and (ii) these antibodies can confer protection against MRSA.

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