MONOCLONAL ANTIBODIES FOR PBP2-a PROTEIN AND HOMOLOGOUS SEQUENCES FOR TREATMENT OF INFECTIONS AND IMMUNODIAGNOSIS ON BACTERIA FROM PHYLUM *FIRMICUTES*

Invention Field

The current invention refers to monoclonal antibodies able to recognize and bind to PBP2a protein and other proteins presenting sequences homologous to PBP2a, including the pathogens methicillin-resistant *Staphylococcus aureus* - MRSA, coagulase-negative *Staphylococcus*, *Staphylococcus sciuri*, *Enterococcus* spp., and any other bacterium possessing PBP2a or sequences homologous to this protein.

The invention still refers to the use of monoclonal antibodies able to recognize and bind to PBP2a protein and other proteins presenting sequences homologous to PBP2a in a complementary immunodiagnosis for detection of resistance to beta-lactams.

Invention Rationales

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are a major concern cause for clinicians, presenting mortality and morbidity rates higher than the ones of infections caused by methicillin-sensitive *Staphylococci* (1). Furthermore, these infections cause longer hospitalization time and higher expense with antimicrobials, leading to a higher cost in treatment of patients infected by this pathogen (1).

Vancomycin has been the first choice antimicrobial for treatment of infections caused by MRSA. However, the growing isolation of MRSA strains in communities in the United States and Australia (2, 3, 4), together with the identification of MRSA strains with intermediate resistance to vancomycin in Japan, United States (5), and Brazil (6), cause the current scene to become more severe. The description of MRSA strains totally resistant to vancomycin in 2004 (7) has caused huge concern in the medical-scientific community. MRSA now represents a strong candidate for becoming the fearful "superbug or superbacterium" - a pathogen resistant to all drugs available nowadays.

Usually, MRSA prevalence rates (ratio of all infections by *S. aureus* caused by MRSA) in hospital infections are gradually increasing in the last decades. In a study conducted by Jarvis *et al.*, including 1268 ICUs (intensive care units) in 337 hospitals in the United States, the number of infections by MRSA in ICUs changed from 660 to 2184 cases and the prevalence increased from 35% to 64,4% (8). In Japan, prevalence rates of hospital infections (HIs) caused by MRSA can present worrying values, from 60% to 90% (9). In studies conducted in the United States, the percentile changed from 2% in 1974 to 50% in 1997 (10, 11) and, in some hospitals in the United States, more than 80% of HIs are caused by MRSA (12). In England, the prevalence increased from 1.5% to 15.2% between 1989 and 1995 and now (2004) the estimate is 41.5% (13).

Besides high prevalence rates, especially in teaching and large-sized hospitals, MRSA is deemed as the main pathogen causing epidemic outbreaks in Brazilian hospitals (14). In 1986, more than 50% of *S. aureus* strains with hospital origin isolated in patients from university hospitals in São Paulo were resistant to methicillin and, in 1993, the incidence of MRSA in the Pediatric Hospital of *Paulista* Medicine School was 70% (15). In a study conducted in hospitals in Belo Horizonte, Resende *et al.* (16) pointed out a prevalence of 71% of MRSA.

MRSA strains present a penicillin-binding protein with very low affinity for antimicrobials in the beta-lactam class, such as PBP2a (17). In the presence of this enzyme, which is codified by gene *mec*A, the bacterium is successful to synthesize the peptidoglycan, even in the presence of beta-lactams. This enzyme can be also found in coagulase-negative *Staphylococcus* and in *Staphylococcus sciuri* - a bacterium present in the normal flora of dogs. Besides resistance to beta-lactams, hospital MRSA strains present resistance to most other available antimicrobial classes, with the use of glycopeptides (vancomycin and teicoplanin) remaining as first choice treatment.

Two studies using DNA vaccine against PBP2a showed that this protein is immunogenic and that the obtained immune response was able to confer protection against MRSA in assays conducted in murine model (18, 19). However, it is known that, in hospital infections, most patients are immunodepressed (20). In this cases, a vaccine would not be able to generate protective antibodies in due time to control a bacterial infection.

PBP2a Immunogenicity

PBP2a is a class II multimodular enzyme according to Goffin and Ghuysen's classification (40). This 76-kilodalton enzyme is composed by a membrane-binding region, a non-transpeptidase domain, and a transpeptidase domain, containing a 4-amino acid active core (STQK), responsible for the bacterial transpeptidation reactions (20 bis Ryfell, 1990).

State of the art studies with the DNA vaccine against PBP2a show that the results of bacterial reduction (renal quantification) in immunized animals subjected to challenge by systemic infection were of 3 to 4 times in the work of Ohwada *et al.* and of 1000 times in the work of Senna *et al.* The authors of these studies used the full sequence (except the membrane fixation region) of the gene *mec*A and an internal fragment of transpeptidase domain, respectively.

But, according to what was mentioned above, a vaccine is not able to generate protective antibodies in due time to control a bacterial infection. Thus, in case of infections by MRSA, the administration of anti-PBP2a monoclonal antibodies is the most proper therapy for treating these infections.

Invention Summary

The main objective of the current invention is to provide monoclonal antibodies able to recognize and to bind to PBP2a protein (SEQ ID NO.:1) and other proteins presenting sequences homologous to PBP2a, including the pathogens methicillin-resistant *Staphylococcus aureus* - MRSA, coagulase-negative *Staphylococcus*, *Staphylococcus sciuri*, *Enterococcus* spp., and any other bacterium possessing PBP2a or sequences homologous to this protein.

Another invention objective is the use of monoclonal antibodies able to recognize and bind to the PBP2a protein and other proteins presenting sequences homologous to PBP2a in a complementary immunodiagnosis for detection of resistance to beta-lactams.

The monoclonal antibodies of the current invention are represented by sequences SEQ ID NO.: 6, SEQ ID NO.: 7, SEQ ID NO.: 8, SEQ ID NO.: 9, SEQ ID NO.: 10,

SEQ ID NO.: 11, SEQ ID NO.: 12, SEQ ID NO.: 13, SEQ ID NO.: 14, SEQ ID NO.: 15, SEQ ID NO.: 16, and SEQ ID NO.: 17.

Brief Figure Description

Figure 1 shows the immunoenzyme test (ELISA) of sera of animals immunized for producing anti-PBP2a antibodies.

Figure 2 shows the result of a polyacrylamide gel with raw samples and samples after purification.

Figure 3 shows the immunoblotting test of MRSA and MSSA lysates against a supernatant containing anti-PBP2a monoclonal antibodies.

Figure 4 is a representation of flow cytometry results of MRSA (CEB) and MSSA bacteria, incubated with anti-PBP2a monoclonal antibody and marked with phycoerythrin (PE).

Figure 5 shows the *in vitro* protection test (MIC - minimum inhibitory concentration) conferred by anti-PBP2a purified monoclonal antibody and vancomycin against an inoculum of different MRSA strains.

Figure 6A shows renal quantification results in treated and non-treated animals with the anti-PBP2a monoclonal antibody, subjected to systemic infection with a sublethal dose of MRSA CEB strain.

Figure 6B shows renal quantification results in treated and non-treated animals with the anti-PBP2a monoclonal antibody, subjected to systemic infection with a sublethal dose of MRSA Iberian strain (European epidemic clone).

Figure 6C shows renal quantification results in treated and non-treated animals with the anti-PBP2a monoclonal antibody, subjected to systemic infection with a sublethal dose of WB79 CA-MRSA strain (Brazilian community strain).

Figure 7A shows the survival curve of treated (protected) and non-treated (control) animals after infection by a lethal bacterial dose (MRSA CEB).

Figure 7B shows the survival curve of treated (protected) and non-treated (control) animals after infection by a lethal bacterial dose (Iberian MRSA).

Figure 7C shows the survival curve of treated (protected) and non-treated (control) animals after infection by a lethal bacterial dose (WB79 CA-MRSA).

Figure 8A shows the bacterial quantification in kidneys of animals treated with anti-PBP2a monoclonal antibody, vancomycin, and antibody + vancomycin association and non-treated animals after infection with bacteria (MRSA CEB).

Figure 8B shows the bacterial quantification in kidneys of animals treated with anti-PBP2a monoclonal antibody.

Figures 9 show the interaction between recombining PBP2a (antigen) and monoclonal antibodies clone 38 (Figure 9A) and clone 10 (Figure 9B).

Figure 10 is a graph of the second analysis by flow cytometry of MRSA samples in presence of FITC-marked anti-PBP2a antibody.

Figure 11 shows the result of the *in vitro* protection conferred by the antibody against VRE enterococcus strain.

Figure 12 refers to LD_{50} and lethal dose for intraperitoneal infection determination.

Figure 13 shows the efficacy result of *in vivo* protection of anti-PBP2a and PBP5 monoclonal antibody from enterococci against systemic infection.

Detailed Invention Description

The emergence of infections by bacteria multiresistant to antimicrobials is presenting an alarming increase. The prevalence of hospital infections caused by MRSA has increased in all world parts present high morbidity and cause high cost due to the intensive use of antimicrobials and to the longer period of patient hospitalization (24). The treatment based on chemotherapy has been showing signs of exhaustion, with appearance of very few really novel and efficient drugs in the market (25).

In this context, passive immunotherapy (re)appears as a promising alternative, presenting a range of advantageous features compared to conventional chemotherapy: among them, the lower toxicity, higher plasma half-life (which facilitates the treatment by dose reduction and maybe a lower final treatment cost), and, especially in the case of the product presented here, selective toxicity, recommended by Paul Ehrlich (*where the drug selectively eliminates the referred pathogen*) (25a). This last feature is extremely useful to prevent the so called selection pressure, played by broad-spectrum antimicrobials - which provides the appearance of multiresistant bacteria (26).

In order to develop this product, the inventors selected PBP2a as target, based on preliminary studies with DNA vaccine in murine models (18, 19, 21) and immunostructural molecule analyses. PBP2a had its structure elucidated in 2002 (29), and is deposited at PDB (Protein Data Bank) under the code lwmr.

Differently from other approaches, the inventors worked with an internal region of the molecule with 76 amino acids, which flank the enzyme active core **(SxxK)**, at the transpeptidase domain. Epitope identification analyses were performed at the molecule and in their presentation to the different class II CHM alleles (Tepitope program), with later verification of their location at the molecule (SPDB-Viewer program). This approach enables us to evaluate the facility of antibody access to these targets at the native molecule.

This computerized analysis showed the presence of epitopes close to the enzyme active core, with excellent recognition degree by class II CHM alleles, located at PBP2a surface (not shown data).

These *in silico* previews were confirmed by the performed target recognition tests (immunoblotting and flow cytometry), and the antibody binding to this PBP2a region showed to be able to confer high protection, demonstrated by the results obtained in the performed *in vitro* and *in vivo* tests. The antibodies generated by the 76-amino acid fragment showed to confer higher protection than the ones generated by immunization with full PBP2a, as previously shown by Ohwada and Senna's works and were confirmed by the results from the inventors.

The epidemiology of infections caused by MRSA shows the presence of the predominant clonal types, responsible for outbreaks and epidemics in hospitals in the whole world. These clones present a higher colonization and virulence capacity when compared to non-epidemic MRSA strains (30).

As a way to validate the obtained results, it was intended to work with different MRSA clones (epidemic clones), representatives from most infections (especially the hospital ones) caused by MRSA.

CEB strain (Brazilian epidemic clone) is responsible for most infections caused by MRSA in Brazilian hospitals (31), being also identified in other countries in South America (32, 33), Portugal, and Czechoslovakia (34). The European epidemic clone (Iberian-MRSA) is found in European countries and in the United States (35).

Due to the increase in cases of community infections caused by MRSA, one of these strains (WB79 CA-MRSA), isolated in Brazil, was included in the current trials. These trials present features differentiated from the ones found in hospitals, with higher virulence (presence of Panton-Valentine leukocidin) and profile of resistance to antimicrobials different from the one of hospital MRSA strains (36). Recently, an outbreak of infections by CA-MRSA in a population of men practicing sex with other men was identified in the United States (37). From this date, we can say that infections caused by MRSA assume a STD (sexually transmitted disease) character.

The protection results conferred by administration of anti-PBP2a antibody showed efficacy against all the different tested clones, which led us to believe in their applicability for infections (community or hospital) caused by any MRSA type.

Some significant features of the product of the current invention need to be commented.

(i) *In vitro* protection results cause to believe that the involved mechanism of action - block of a region close to the molecule active core - is sufficient to inhibit the bacterial growth and multiplication. Similarly to the one of beta-lactam antibiotics, this mechanism is time-dependent, needing that the bacterium is in the multiplication phase to expose the target to the drug action.

(ii) This feature is favored by the pharmacokinetic properties of antibodies, which present long plasma half-life (38). Comparing to the antibiotic therapy, this means an administration in lower number of doses, presenting treatment facility for the patient and maybe lower final cost as consequences. These ideas were confirmed in practice in the protection comparative assay with vancomycin (see below).

(iii) The inhibition of *in vitro* bacterial growth also means that the antibody does not need the traditional ancillary antigen-antibody response mechanisms, such as opsonization and complement activation. Considering that most hospital infections occur in patients that has immunodepression status, this product feature is extremely significant.

(iv) It is known that *Staphylococcus aureus* presents a protein A on its surface. This molecule characteristically binds itself to the Fc region of immunoglobulins, impeding the opsonizing activity of the immune system (38). Due to the results obtained in our trials, we believe that the administered antibody concentration was able to saturate the protein A existing at the bacterial surface, not preventing the PBP2a block by the administered antibodies.

Systemic infections caused by MRSA have been treated with use of glycopeptides, especially vancomycin. However, this drug presents a range of side effects, with immunotoxicity (42), ototoxicity, nephrotoxicity, and transient neutropenia (43) among them, and need administration for long periods, presenting high final treatment cost.

By using a murine infection model, it was possible to compare a treatment with vancomycin versus monoclonal antibody and the combination of the two drugs. The obtained results indicated that an antibody dose had action similar to or higher than 5 vancomycin doses and that the simultaneous administration of the two drugs was very more efficient in eliminating the bacterium than the isolated administration. These data are extremely significant, as it is possible to think about a lower treatment cost with the antibody and a more efficient recovery of patients with severe status with the administration of vancomycin and antibody.

In addition to the use of anti-PBP2a monoclonal antibodies for treating infections by MRSA, the current invention still considers the following applications:

(i) the results of recognition of a protein with molecular weight close to the one of PBP5 in *Enterococcus* sp. strain by anti-PBP2a monoclonal antibody indicate that it is possible to obtain a protective response against this pathogen with the antibody administration. PBP5 presents homology with PBP2a, with both being classified as class B multimodular PBPs, with low affinity for beta-lactams (40). Enterococci are bacteria that cause severe hospital infections, presenting high degree of intrinsic and acquired resistance to antimicrobials. Strains resistant to vancomycin (VRE) represent a reservoir of genes of resistance to glycopeptides and can be transferred to other pathogens (41).

(ii) these antibodies can have applicability for PBP2a identification by immunodiagnostic tests. For example, the use of immune tests based on agglutination of latex particles bound to the antibody can provide a result that foresees resistance to all beta-lactam antibiotics in few hours. In the conventional tests of sensitivity to antimicrobials (such as antibiotic sensitivity testing), these results are only released after 12 to 24 hours.

(iii) once monoclonal antibodies have been successfully used in topic applications against chronic sores (infliximab, see reference Streit *et al.*, and WO 1999041285 A1), these antibodies might be topically administered to promote nasal decolonization by MRSA and for treatment of skin lesions caused by this pathogen.

Based on the knowledge and issues found in the state of art, the current inventors performed immunizations in animals with a DNA vaccine with constructions corresponding to the gene *mec*A (SEQ ID NO.: 2) without the membrane fixation region and immunizations with a 76-amino acid internal region of the transpeptidase domain (SED ID NO.: 3) comprising the enzyme active core.

The immunizations were performed in the same conditions presented in the works of Ohwada *et al.* and Senna *et al.*, who developed a DNA vaccine against PBP2a, using the full sequence (except the membrane fixation region) of the gene *mec*A and an internal fragment of the transpeptidase domain. The immunizations were performed with 4 doses, and the immunized animals and a non-immunized control group were challenged by a systemic infection with MRSA and a determination of the number of bacteria present in the kidneys of animals in two distinctive periods. The obtained results showed that the immunization with the transpeptidase fragment conferred a greater reduction in the number of bacteria present in the animal kidneys than the one in the animals immunized with gene *mec*A.

Thus, we see that the antibodies generated against the transpeptidase fragment provided a better protection in the used conditions (DNA vaccine in murine model) than the one by antibodies against PBP2a. The transpeptidase fragment includes the enzyme active core STQK (SEQ ID NO.: 4).

Consequently, based on the found results, the inventors directed the invention for producing monoclonal antibodies able to recognize and bind to the PBP2a protein and other proteins presenting sequences homologous to PBP2a protein and using the transpeptidase fragment that includes the enzyme active core.

The invention will be now described with reference to examples, which must not be considered as limiting.

MATERIALS AND METHODS:

1. Bacteria:

The following methicillin-resistant *Staphylococcus aureus* strains were used: Iberian-MRSA, COL-MRSA (ceded by *Unité des Agents Antimicrobiens - Institut Pasteur* [Unit of Antimicrobial Agents - Pasteur Institute], Dr. Patrice Courvalin), WB79 CA-MRSA, and CEB-MRSA (ceded by Dr. Agnes Figueiredo, *Instituto de Microbiologia* [Microbiology Institute] of UFRJ); a vancomycin-resistant *Enterococcus faecalis* strain; and a methicillin-sensitive *Staphylococcus aureus* (MSSA) strain. *Escherichia coli* strains BL21 DE3 (Novagen) and TOP10 (Invitrogen) were also used as control.

2. Animals:

Female Balb/C mice, 4 to 8 weeks old, obtained from CECAL-FIOCRUZ and housed at LAEAN-BioManguinhos were used in the immunization and *in vivo* protection assays.

3. Immunization:

The mice (4 animals) received an initial dose of 100 micrograms of pCI-Neo plasmid: fragment of MRSA *mec*A gene (18), followed by a 10-microgram dose of purified recombining protein 14 days later, corresponding to an internal region of the MRSA PBP2a (21), emulsified in complete Freund adjuvant, followed by other dose 14 days later, emulsified in incomplete Freund adjuvant. Fourteen days later, the animal with best immune response (evaluated by enzyme immunoassay - ELISA) received an intravenous dose of 10 micrograms of purified protein diluted in PBS (phosphate buffered saline). Three days after the IV injection, the animal was subjected to euthanasia by asphyxia in CO_2 (**CEUA L0009-07 Protocol - FIOCRUZ**), and the spleen was aseptically removed for cell fusion. The serum of the animal with best immune response was used as positive control for other immunological tests.

4. Production of monoclonal antibodies:

Lymphocytes removed from spleen were subjected to fusion with SP2/0-Ag14 myeloma cells (ATCC 1581), using polyethylene glycol for fusion, and were cultured in hypoxanthine-aminopterin-thymidine medium at 37°C in atmosphere of 10% CO_2, according to the protocol for producing monoclonal antibodies in *Current Protocols in Immunology* (22). The resulting hybridomas were evaluated by ELISA test after 14 days, using the purified recombining protein as antigen, according to what is described below. The best hybridomas were subjected to cloning, with selection of the best clones by ELISA and these latter being stored in liquid nitrogen.

5. Enzyme immunoassay - ELISA:

Maxisorb 98-well plastic plates were sensitized with 500 nanograms/well of recombining protein (PBP2a fragment) in carbonate/bicarbonate buffer and incubated at 4°C for the whole night. In the following day, the plates were washed three times with PBS containing 0.05% of Tween 20 and blocked in PBS and 5% skimmed milk for two hours at 37°C. The samples to be analyzed (serum of immunized animals diluted at 1:100 or cell culture supernatants) and incubated for 2 hours at 37°C were used. The

plate was washed three times with PBS and Tween 20 (0.05%) again, and the anti-Ig conjugate (anti-mouse HRP Ig SIGMA A 0412) was added at 1:5000 dilution, followed by incubation at 37°C for 90 minutes. After this period, the plate was washed three times with PBS and Tween 20 (0.05%), with addition of TMB peroxidase color developer (BioRad) and incubation for 15 minutes protected from light. The reaction was interrupted by addition of 0.5 N H_2SO_4, and the reading was performed at 450 nm. A 1:200 diluted hyperimmune polyclonal serum was used as positive control.

5.1. Avidity assays based on enzyme immunoassay

5.1.1. Avidity assay with urea (Niederhouser *et al.* - 5.1):

The protocol is similar to the immunoassay one (5), with the following modifications: after the sample incubation (100 ng of clone 10 purified monoclonal antibody and 2.0 ng of clone 38 purified monoclonal antibody) for two hours at 37°C, the samples were subjected to three washings with 8 M urea in PBS and Tween 20 (0.05%), followed by four washings in PBS and Tween 20 (0.05%), presenting the same normally processed sample (without treatment with urea) as control. After the reading of sample optical densities, the avidity rate was calculated by the ratio of the reading with urea divided by the reading without urea, multiplied by 100 (percentile result).

5.1.2. Avidity assay with ammonium thiocyanate (Goldblat *et al.* - 5.2)

The protocol is similar to the immunoassay one (5), with the following modifications: after the sample incubation (100 ng of clone 10 purified monoclonal antibody and 2.0 ng of clone 38 purified monoclonal antibody) for two hours at 37°C, the samples were subjected to treatment with ammonium thiocyanate for 30 minutes at 37°C in the following concentrations: 3 M; 1.5 M; 1.0 M; 0.75 M; 0.50 M; 0.25 M; and 0.125 M. A sample non-treated with ammonium thiocyanate was used as control for each clone.

After the reading of sample optical densities, the avidity rate was calculated by the following formula: **AR** (avidity rate) = [(log 50 - log **A**) x (**B** - **A**)/log **B** - log **A**] + **A**; where log 50 = 1.70. **A** is the lowest ammonium thiocyanate concentration that results in an absorbance reduction lower than 50%, and **B** is the highest ammonium thiocyanate concentration that results in an absorbance reduction higher than 50%.

6. Monoclonal antibody production and purification:

A sample of previously selected clones 10 and 38 was grown in a serumless medium (GIBCO VP-SFM) with addition of 1% BSA in 100-mL vials in a stove with atmosphere of 10% CO_2. The supernatants were subjected to centrifugation, followed by filtration in 0.22-micrometer filters and purified in high performance chromatography (HPLC) with SelectSure protein A MAB resin (GE). The antibodies were neutralized at pH 7.0 with 1 M Tris, pH 10.0, dialyzed against PBS 0.5x in deionized water. The samples were subjected to lyophilization process, resuspended in deionized water, quantified by the Lowry method, and evaluated by electrophoresis in polyacrylamide gel.

7. Target recognition:

7.1. *In vitro* PBP2a recognition - immunoblotting:

A MRSA (CEB) strain, a methicillin-sensitive *Staphylococcus aureus* (MSSA) strain, a vancomycin-resistant *Enterococcus faecium* strain, and the BL-21 DE3 *Escherichia coli* strain were grown in exponential phase. One mL of each sample was centrifuged and lysed by agitation in glass beads in a mini-Bead Beater device (Biospect Products), 3 times for 30 seconds. One aliquot of each sample was subjected to electrophoresis in 12% denaturing polyacrylamide gel (SDS-PAGE), and the proteins were later transferred to a nylon membrane (Hybond N-BioRad). The membrane was blocked under mild agitation for two hours in PBS buffer containing 10% skimmed milk and 1% BSA (bovine serum albumin). The membrane was washed three times in PBS and Tween 20 (0.05%) and three times in PBS. Then, the latter was placed in incubation for two hours, with supernatant of anti-PBP2a monoclonal antibody diluted in PBS at 1:1 ratio. After the incubation, the membrane was washed as previously described, and the alkaline phosphatase conjugate (murine anti-IgG antibody - Sigma A3688) at 1:15,000 ratio and incubated for ninety minutes. After this period, the washing in PBS was performed again, and the development with Western Blue substrate for alkaline phosphatase (Promega) was performed.

7.2. PBP2a recognition at the bacterium surface - flow cytometry:

A MRSA (CEB) strain was grown in stationary (ON) or exponential phase. The samples were washed in PBS 1x and resuspended at a DO_{600} of 0.6 (~10^8 bacteria/mL). These latter were centrifuged in 1 mL (10^8 bacteria) and resuspended in 0.5% BSA and a 1:10 dilution of normal serum (murine/human). Then, the samples were incubated for 30 min at 4°C, and later, the concentrates (pellets) were washed 2 times in PBS and resuspended in 100 microliters of PBS containing 0.5% BSA and a 1:10,000 dilution of anti-PBP2a monoclonal antibody and incubated for 30 minutes at 4°C. After this step, the samples were washed as previously again and resuspended in 100 microliters of PBS and 0.5% BSA and a dilution (1:1000) of PE (phycoerythrin) mouse anti-Ig conjugate, later being incubated at dark for 30 min at 4°C. Then, the samples were washed again and fixated for 15 minutes at 4°C in PBS containing 2% paraformaldehyde. After this preparation, the samples were analyzed in a flow cytometer (Becton and Dickinson - FACScalibur).

8. *In vitro* protection assays - determination of minimum inhibitory concentration:

MSRA strains (CEB, Iberian, COL, and CA) were grown in exponential phase up to 600 nm of optical density equal to 0.5. The applied inoculum was adjusted for containing approximately 100,000 bacteria. Müller Hinton broth, bacterial inoculum, and growing amounts of purified anti-PBP2a monoclonal antibody were added to test tubes or 24-cavity plates. The plates or tubes were subjected to incubation at 37°C for 12 hours. After this period, presence or absence of turbidity was observed in the samples. The minimum inhibitory concentration was considered as the lower antibody amount able to inhibit the bacterial inoculum growth (100,000 bacteria).

9. *In vivo* protection assays:

9.1. Determination of lethal dose and LD_{50} for MRSA strains.

The determination of lethal dose and LD_{50} were performed according to the Reed-Muench method (23) for MRSA strains (CEB, Iberian, WB79 CA, and COL). Groups of 8-week female Balb/C mice were inoculated by intraperitoneal route with growing bacteria doses and observed for 7 days. The animals that survived after this period were subjected to euthanasia, according to the established animal welfare rules.

9.2. Systemic infection and bacterial renal quantification assays.

MRSA strains (CEB, Iberian, and WB79 CA) were grown in exponential phase (DO_{600} ~0.6), washed and resuspended in sterile PBS 1x at DO_{600} ~0.5, corresponding to approximately 2 x 10^8 bacteria. This concentration was calculated by dilution and seeding on BHI agar plates containing 10 micrograms of oxacillin/mL. Female 8-week Balb/C mice received one intraperitoneal dose of 400 micrograms of purified anti-PBP2a monoclonal antibody in the first day. In the sixth day, the animals were subjected to euthanasia and the kidneys were aseptically removed. Then, the kidneys were homogenized in 1 mL of sterile Luria broth and successively diluted in series of 10. One hundred microliters of each dilution were seeded on BHI agar plates containing 10 micrograms/mL of oxacillin and incubated for 24 hours at 37°C, with a count of resulting colonies and calculations of total dilutions being performed.

9.3. Survival assay.

MRSA strains were grown in the same previously described conditions, but an inoculum adjustment was performed for the previously established LD_{50}. Female 8-week Balb/C mice received one intraperitoneal dose of 500 micrograms of purified anti-PBP2a monoclonal antibody in the first day. In the following day, these animals plus a control group were infected with one dose of about 2.5 to 6.0 x 10^8 bacteria by intraperitoneal route, according to the LD_{50} of each strain. The animals were observed for 10 days, with the survivors being subjected to euthanasia.

10. Comparative *in vivo* protection study of anti-PBP2a monoclonal antibody and vancomycin - renal quantification:

Assay I

Four groups of female 8-week Balb/C mice (4 animals per group) received an infective dose of 6.0 x 10^7 bacteria (CEB-MRSA) by intraperitoneal route. The animals received doses of purified monoclonal antibody (MAB), vancomycin, MAB + vancomycin, and negative control, according to the groups below:
group 1: MAB (400 micrograms) (first day)
group 2: vancomycin (150 micrograms, intramuscular route, 12/12 hours)
group 3: vancomycin + MAB (350 micrograms) (1 day after infection)
group 4: control
The first doses of antibody and vancomycin were administered 4 hours after the administration of the infective dose. The animals were subjected to euthanasia in the

fourth day, and the kidneys were aseptically removed and subjected to renal qualification, according to what was previously described.

Assay II

This assay was performed in the same way as the previous one, but, with a lower infective dose (7.0×10^6 bacteria), with the group 1 being treated with 500 micrograms of purified monoclonal antibody; group 2 being treated with vancomycin (150 micrograms, intramuscular route, 12/12 hours; 5 doses); group 3 being treated with vancomycin + 500 micrograms of monoclonal antibody; and group 4 being control (non-treated animals).

11. Identification complementarity-determining regions (CDRs) of light and heavy chains of the anti-PBP2a monoclonal antibody

11.1. mRNA extraction from hybridoma cells

A 10-mL centrifugate (pellet) of a cell culture of monoclonal antibody-producing hybridoma was processed for mRNA extraction using the RNeasy Minikit kit (Qiagen).

11.2. cDNA obtainment

Reverse transcriptase reaction: M-MLV reverse transcriptase kit (Invitrogen) was used for obtaining complementary DNA, following the manufacturing guidelines.

11.3. Amplification of VH and VL chains by polymerase chain reaction (PCR)

The reactions were performed using the starter sequences (*primers*) below.

SEQ ID NO.	Sequence	Use
	FOR HEAVY CHAIN	
18	5′ ATG(GA) A(GC)TT(GC)(TG)GG(TC)T(AC)A(AG)CT(GT)G(GA)TT 3′	VH Amplification
19	5′ ATG(GA)AATG(GC)A(GC)CTGGT(CT)(TA)T(TC)CTCT 3′	VH Amplification
20	5′ GATGTGAAGCTTCAGGAGTC 3′	VH Amplification
21	5′ CAGGTGCAGCTGAAGGAGTC 3′	VH Amplification
22	5′ CAGGTGCAGCTGAAGCAGTC 3′	VH Amplification
23	5′ CAGGTTACTCTGAAAGAGTC 3′	VH Amplification
24	5′ GAGGTCCAGCTGCAACAATCT 3′	VH Amplification

25	5′ GAGGTCCAGCTGCAGCAGTC 3′	VH Amplification
26	5′ CAGGTCCAACTGCAGCAGCCT 3′	VH Amplification
27	5′ GAGGTGAAGCTGGTGGAGTC 3′	VH Amplification
28	5′ GATGTGAACTTGGAAGTGTC 3′	VH Amplification
29 (gamma 1)	5′ TGGACAGGGATCCAGAGTTCCAGGTCACT 3′	VH Amplification
	FOR LIGHT CHAIN	
30	5′ GACATTGTGATGACCCAGTCT 3′	VL Amplification
31	5′ GATGTTTTGATGACCCAAACT 3′	VL Amplification
32	5′ GATATTGTGATAACCCAG 3′	VL Amplification
33	5′ GACATTGTGCTGACCCAATCT 3′	VL Amplification
34	5′ GATATTGTGCTAACTCAGTCT 3′	VL Amplification
35	5′ GATATCCAGATGACACAGACT 3′	VL Amplification
36	5′ GACATCCAGCTGACTCAGTCT 3′	VL Amplification
37	5′ CAAATTGTTCTCACCCAGTCT 3′	VL Amplification
38	5′CAGGCTGTTGTGACTCAGGAA 3′	VL Amplification
39 (kappa 18)	5′ TACAGTTGGTGCAGCATC 3′	VL Amplification

11.4. Sequencing of light and heavy chains of anti-PBP2a monoclonal antibody

Sequencing Steps:

I. Amplification

This was performed using the previous starter sequences, defined by SEW ID NO.: 18 to SEQ ID NO.: 39.

II. Sequencing

The device ABI Prism 3100, Genetic Analyser (Hitachi) was used.

11.5. Sequence analysis and identification of light and heavy chain CDR

The obtained DNA sequences were analyzed with the aid of DNA Star program, and were translated to amino acid sequences (ExPASy site - Translate program) for later analysis by Kabat's (24) and Chotia's (25) algorithms for identification of light and heavy chain CDRs.

12. Determination of association and dissociation constants for monoclonal antibodies (clones 10 and 38 by surface plasmon resonance [SPR] [Biacore] method)

SPR measurements were performed using a CM-5 sensor in a Biacore X® (Biacore AB, Uppsala, Sweden) device. Binding reagents and HBS-EP buffers (10 mM hepes, 150 mM NaCl, 3 mM EDTA, 0.005% P20 [Tween 0, pH 7.4]) were acquired with the company GE Healthcare.

Example 2

1. Obtainment of murine anti-PBP2a monoclonal antibodies:

A group of animals was subjected to the immunization protocol according to what was previously described. The result evaluated by ELISA test is described in Figure 1.

After the fusion process (fusion of number 90-LATAM), the supernatant from 96 cavities (hybridomas) was analyzed by ELISA test. From this total, the five best samples were selected and the cells were expanded (cloning). Then, again, the resulting supernatants were analyzed by ELISA. Positive samples were validated by immunoblotting against the purified recombinant protein (PBP2a) for validating the results obtained by ELISA. The final result is in Table I.

Figure 1 reveals the result from the immunoenzyme test (ELISA) of the sera of the immunized animals for producing anti-PBP2a antibodies. Each dashing corresponds to the 1:100 diluted serum of the immunized animals. Positive control serum is in angular dashing [⊓⊔⊔]. First bar of each dashing: pre-immune serum; second bar: serum after fourth immunization; and third bar: serum after fifth immunization.

Table I. Clonings of fusion 90: final balance

Cloned hybridomas	Total number of clones	Positive clones (ELISA)	Positive clones (immunoblotting)	Selected clones
77	70	2	1 (clone 38)	1
68	108	10	1 (clone 10)	1
79	91	Zero	-	-
17	101	Zero	-	-
70	48	2	Zero	-
	418	14	2	2

From the selected clones, clone 77-38 was subjected to recloning process for verifying the cell stability for secreting monoclonal antibodies. From the 50 analyzed

cavities, all presented positive result by ELISA test. These clone were expanded and are stocked in liquid nitrogen at LATAM (Laboratory of monoclonal antibody technology) facilities.

2. Growth, production, and purification of monoclonal antibodies:

The process was standardized according to what was previously described. The obtained output is approximately 4 milligrams of monoclonal antibody for every 100 mL of supernatant subjected to the purification process. The obtained results can be seen below.

In Figure 2, we can see a polyacrylamide gel with raw samples and after purification. This Figure 2 shows the non-denaturing polyacrylamide gel with supernatant samples before (column 1) and after purification (2) in HPLC column with MAb SelectSure resin. Columns 3 to 9 correspond to obtained purified sample fractions. The arrow indicates the approximate size of 150 kDa.

3. Functional characterization of monoclonal antibodies:

3.1. *In vitro* PBP2a recognition - immunoblotting

In order to investigate the capacity for antibody recognition by the target in the pathogen (PBP2a and similar sequences), the immunoblotting test was performed with bacteria presenting PBP2a (CEB and COL MRSA), a MSSA (methicillin-sensitive *Staphylococcus aureus*) strain (not presenting PBP2a), a vancomycin-resistant *Enterococcus* sp. strain (presenting PBP5), a transpeptidase with low-affinity for beta-lactam antibiotics (homologous to PBP2a), and an *Escherichia coli* strain (where the recombinant protein was generated) as negative control. The obtained results show that the antibody was able to recognize a protein with molecular weight of approximately 76 kDa in MRSA and *Enterococcus* sp. strains, corresponding to the size of PBP2a and PBP5, respectively. No reactivity of the monoclonal antibody was seen with proteins of methicillin-sensitive *Staphylococcus aureus* (PBP2a-negative) neither with *Escherichia coli* strain. The results can be seen in Figure 3 (immunoblotting against MRSA and MSSA).

The result of the immunoblotting test of MRSA and MSSA lysates against the supernatant containing anti-PBP2a monoclonal antibodies is shown in Figure 3.

1. - molecular weight marker (Kaleidoscope);
2. - MSSA sample grown in exponential phase;
3 and 4. - MRSA sample grown in exponential phase;
5. - MSSA sample grown in stationary phase (overnight); and
6 and 7. - MRSA samples grown in stationary phase. The left arrow indicates PBP2a molecular weight.

3.2. PBP2a recognition on the bacterium surface - flow cytometry

The objective of the flow cytometry test is to validate the capacity for target recognition on the bacterium in its native form by the monoclonal antibody. In previous tests (immunoblotting), we observed the target recognition on proteins subjected to a denaturation process, which occurs during the protein separation by electrophoresis in

denaturing polyacrylamide gel. Again, we analyze a negative control strain (MSSA, PBP2a-negative) and a MRSA strain (CEB) grown in exponential and stationary phases. The obtained results show that the antibody is able to recognize the target on the bacterial surface in both conditions. The presence of protein A on *Staphylococcus* surface was not able to inhibit the antibody binding with PBP2a. The obtained results can be seen in Figure 4.

Figure 4 shows the results of the flow cytometry of MRSA (CEB) and MSSA bacteria incubated with anti-PBP2a monoclonal antibody and marked with phycoerythrin (PE). In (1), MSSA bacteria; in (2), MRSA grown in stationary phase; in (3), MRSA grown in exponential phase. MRSA populations present a right shift, corresponding to an increase in marked cells by the fluorescent conjugate.

4. Evaluation of protection conferred by anti-PBP2a monoclonal antibody:

4.1. *In vitro* protection tests

Determination of minimum inhibitory concentration (MIC)

These assays aim to evaluate the antibody capacity for directly binding itself to the target in a closed system. For monoclonal antibodies with therapeutic finality, positive results have extreme significance, as they mean that the antibody is able to recognize the target and to block the bacterial growth without the aid of the host immune system, such as complement activation and opsonization mechanisms, resulting from the combined action with the innate and adaptative immune system of the host. CEB, COL, and Iberian MRSA strains were evaluated, where similar MIC values (approximately 500 micrograms) were seen in the evaluated conditions. These data indicate that regardless of the genetic background of the different MRSA strains, the antibody doses needed for blocking the growth are not the same. These results can be seen in Figure 5.

Figure 5 shows the test of the *in vitro* protection (MIC - minimum inhibitory concentration) conferred by anti-PBP2a purified monoclonal antibody and vancomycin against an inoculum of 10^5 cells from different MRSA strains. An absence of turbidity indicates that there was no bacterial growth in the analyzed conditions.

1A: CEB MRSA (Brazilian epidemic clone) + 250 μg of antibody;
2A: CEB MRSA + 500 μg of antibody;
3A: CEB MRSA + 750 μg of antibody;
4A and **6A:** negative controls of CEB MRSA strain.
1B: COL MRSA + 250 μg of antibody;
2B: COL MRSA + 500 μg of antibody;
3B: COL MRSA + 750 μg of antibody;
4B and **6B:** negative controls of COL MRSA strain.
1C: Iberian MRSA (European epidemic clone - EEC) + 250 μg of antibody;
2C: EEC MRSA + 500 μg of antibody;
3C: EEC MRSA + 750 μg of antibody;
4C and **6C:** negative controls of EEC MRSA strain.
1D: CEB MRSA + 150 μg of vancomycin;
2D: CEB MRSA + 300 μg of vancomycin;
3D: CEB MRSA + 500 μg of vancomycin;

4D: CEB MRSA + 750 μg of vancomycin;
5D and **6D:** negative controls.

4.2. *In vivo* protection tests

4.2.1. Determination of lethal and sublethal doses for CEB, Iberian, WB79 CA, and COL MRSA strains:

These assays were necessary in order that the *in vivo* protection was evaluated in the two used models - the renal quantification by sublethal dose one and the survival test after systemic infection with a larger inoculum of bacteria - able to cause death in about 50% of the animals (LD_{50}). The chosen route was intraperitoneal, due to the administration facility and the absence of losses. An adaptation of Reed-Muench method was used for conducting the assay, with two animals per condition (infective bacterial dose in growing concentrations) for determining LD_{50} and sublethal doses, and three different doses were tested, once we had previous knowledge on the mean lethal doses for *Staphylococcus aureus*. COL MRSA strain, the first MRSA clone to have its genome sequenced, is used as reference strain for studies on this pathogen. However, it showed to be little virulent, needing high infective doses in relation to the other MRSA clones to cause infection in animals. Therefore, it was not used in protection assays.

4.2.2. Renal protection assays after systemic infection with sublethal dose

Using a renal quantification model after infection, these assays enable to evaluate the antibody capacity to reduce the presence of bacteria in vital organs (kidneys) after a systemic infection. A reduction higher than 3 log (1000 times) was reached in three independent assays with virulent MRSA strains from different genetic backgrounds. In these assays, the animals received a previous dose of 500 micrograms of antibody. The protection conferred by a lower antibody dose (250 micrograms) was evaluated in the assay with CA-MRSA strain, where a bacterial reduction was also observed, but lower than the one obtained with the 500-microgram dose. The results can be seen in Figures 6A, 6B, and 6C.

Figure 6A shows the results of renal quantification in animals treated and non-treated with anti-PBP2a monoclonal antibody and subjected to systemic infection with a sublethal dose of MRSA CEB strain. In horizontal stripes: log of concentration of bacteria isolated from the kidneys of each non-treated animal. In checkered pattern: log of quantity of bacteria isolated from the kidneys of each animal treated with the antibody. Bacterial quantification: controls: C1: 2000 bacteria; C2: 29,000 bacteria; C3: 220,000 bacteria; C4: 52,000 bacteria (mean of 75,750 bacteria). Treated (protected) animals: P1: 20 bacteria; P2, P3, and P4: 10 bacteria (mean of 12.5 bacteria). Reduction in the quantity of bacteria recovered from treated animals in relation to non-treated ones: 6060 times.

Figure 6B shows the results of renal quantification in animals treated and non-treated with anti-PBP2a monoclonal antibody and subjected to systemic infection with a sublethal dose of Iberian MRSA strain (European epidemic clone). In horizontal stripes: log of concentration of bacteria isolated from the kidneys of each non-treated animal. In large checkered pattern: log of quantity of bacteria isolated from the kidneys of each animal treated with the antibody. Bars in small checkered pattern indicate the respective

obtained means. Bacterial quantification: controls: C1: 210,000 bacteria; C2: 44,000 bacteria; C3: 300,000 bacteria; C4: 290,000 bacteria (mean of 211,000 bacteria). Treated (protected) animals: P1: 80 bacteria; P2: 200 bacteria; P3: 10 bacteria; and P4: 60 bacteria (mean of 87.5 bacteria). **Reduction in quantity of bacteria recovered from treated animals in relation to the non-treated ones: 2420 times.**

Figure 6C shows the results of renal quantification in animals treated and non-treated with anti-PBP2a monoclonal antibody and subjected to systemic infection with a sublethal dose of WB79 CA-MRSA strain (Brazilian community strain). Five first bars (in xx, horizontal dashing, and large checkered pattern): log of concentration of bacteria isolated from the kidneys of each non-treated animal. First bar (in xx): estimate in relation to an animal killed before the euthanasia. Bars 6, 7, 8, and 9 (in horizontal dashing and checkered pattern): log of quantity of bacteria isolated from the kidneys of animals treated with 250 μg of anti-PBP2a monoclonal antibody. Bars 10, 11, 12, and 13 (in triangles): log of quantity of bacteria isolated from the kidneys of each animal treated with 500 μg of antibody. Checkered bars (5^{th}, 9^{th}, and 13^{th} bars) indicate the respective obtained means. Bacterial quantification: controls: C1: 650,000 bacteria; C2: 26,000 bacteria; C3: 17,000 bacteria; C4: 500,000 bacteria (*dead animal estimate*) (mean of 231,000 bacteria). Animals treated with 250 μg of antibody: P1: zero; P2: 5,400 bacteria; P3: 830 bacteria; and P4: 10 bacteria (mean of 1,560 bacteria). Animals treated with 500 μg of antibody: P1: 80; P2: zero; P3: 210; P4: 80 bacteria (mean of 92.5), **Reduction in quantity of bacteria recovered from treated animals in relation to the ones non-treated with 250 μg of antibody: 149 times. With 500 μg: 2,497 times.**

4.2.3. Survival assays

In this assay type, we evaluate the protection conferred by the antibody to animals after an infection with a bacterial load able to kill 50% of the animals (LD_{50}) or more. The protection against the three strains used in the renal quantification assay was evaluated. A significant reduction in (i) survival time and (ii) the very survival of animals under treatment with anti-MRSA monoclonal antibodies was observed in the three independent assays.

In the assay with CEB MRSA strain, 70% of the animals under treatment survived the infection, against only 10% of the control group (non-treated). In the assay with Iberian MRSA strain, the obtained results were similar, with 60% of protection in animals under treatment and 100% of death in control group. In the assay with CA MRSA strain, a protection of 100% was seen, compared to 70% in control animals. These results can be seen in Figures 7a, 7b, and 7c.

Figure 7A shows the survival curve of treated (protected) and non-treated (control) animals after infection by a dose of 2.3×10^8 bacteria (CEB MRSA) administered by intraperitoneal route (LD_{50}).

Figure 7B shows the survival curve of treated (protected) and non-treated (control) animals after infection by a dose of 4.2×10^8 bacteria (Iberian MRSA) administered by intraperitoneal route (~LD_{50}).

Figure 7C shows the survival curve of treated (protected) and non-treated (control) animals after infection by a dose of 1.1×10^9 bacteria (WB79 CA-MRSA) administered by intraperitoneal route (~LD_{50}).

4.2.4. Comparative study on protection conferred by monoclonal antibody versus vancomycin

As vancomycin is a first choice antimicrobial for treatment of severe infections by MRSA, a comparative study on protection was conducted in a model different from the previous ones. In this study, the animals were infected and the administration of the antimicrobial or monoclonal antibodies was started just after four hours of infection. The study was conducted with three distinct groups: one treated with vancomycin, other treated with monoclonal antibodies, and a third one with simultaneous administration of antimicrobial + antibodies. Vancomycin doses were adjusted and administered in a way similar to the cases of infections in humans (500 mg every 12 hours).

The obtained results indicated that there was a reduction of around 15 times in the quantity of bacteria present in the kidneys of animals treated with antimicrobial or antibodies three days after the infection. However, a reduction of 4,617 times was seen in the group that received treatment with antimicrobial and antibodies.

Based on these results, we can conclude that the protection conferred by an antibody dose corresponded to five vancomycin doses and that the simultaneous administration of vancomycin and antibodies was very efficient in reducing the bacterial load observed in the kidneys of infected animals. This study was repeated with a little lower infective dose in order that we could better observe the protective action of anti-MRSA monoclonal antibodies both isolated and in association with vancomycin (See figures 8).

After the conduction of the second assay with a lower infective dose, the obtained results confirmed the initial results. The protection conferred by the treatment with monoclonal antibody caused a reduction of 89 times, which was higher than the protection obtained with the treatment with 5 vancomycin doses (reduction of 35 times). However, the most significant reduction result was seen in the group treated with antibody + vancomycin, causing a reduction of 450 times.

Figure 8A shows the bacterial quantification in kidneys of animals treated with anti-PBP2a monoclonal antibody, vancomycin, and association of antibody + vancomycin and in non-treated animals after infection with 6.0×10^7 bacteria (CEB MRSA). The treatment beginning happened 4 hours after the infection. Vancomycin was administered every 12 hours (5 doses). Bars 1, 2, 3, 4, and 5 (dashing): log of concentration of bacteria recovered from non-treated animals (controls). C1: 7,000,000; C2: 295,000; C3: 380,000; C4: 3,200,000 (mean: 2,718,750 bacteria). Bars 6, 7, 8, 9, and 10 (checkered pattern): log of concentration of bacteria recovered from animals treated with 400 μg of anti-PBP2a monoclonal antibody. P1: 4,200; P2: 310,000; P3: 330,000; P4: 90,000 (mean of 183,550 bacteria). Bars 11, 12, 13, 14, and 15 (spheres): animals treated with vancomycin. P1: 110,000; P2: 58,000; P3: 500,000; P4: 21,000 (mean of 172,250 bacteria). Bars 16, 17, 18, 19, and 290 (triangles): log of concentration of bacteria recovered from animals treated with antibody (300 μg) + vancomycin. P1: 1,100; P2: 700; P3: 450; P4: 90 (mean of 585 bacteria).

Figure 8B shows the bacterial quantification in kidneys of animals treated with anti-PBP2a monoclonal antibody (bars 7 to 12), vancomycin (bars 13 to 18), antibody + vancomycin association (19 to 24) and of non-treated animals (bars 1 to 6) after infection with 7.0×10^6 bacteria (CEB MRSA). Treatment beginning in 4 hours after infection. Vancomycin administered every 12 hours (5 doses). Bars 1 to 6: log of concentration of bacteria recovered from non-treated animals (controls). C1: 6,000; C2:

1,000; C3: 500; C4: 118,000; and C5: 1,000 (mean: 25,220 bacteria). Bars 7 to 12: log of concentration of bacteria recovered from animals treated with 500 μg of anti-PBP2a monoclonal antibody. MB1: 450; MB2: 200; MB3: 100; MB4: 20; MB5: zero (mean of 284 bacteria). Bars 13 to 18: animals treated with vancomycin. VC1: 100; VC2: 700; VC3: zero; VC4: zero; VC5: 2800 (mean of 720 bacteria). Bars 19 to 24: log of concentration of bacteria recovered from animals treated with antibody (500 μg) + vancomycin. MBV1: 130; MBV2: 20; MBV3: 10; MBV4: 80; MBV5: 20 (mean of 56 bacteria).

5. Avidity assays

Results of the avidity tests of monoclonal antibodies clones 10 and 38:

Urea protocol avidity:
clone 10: 1.03/1.46 (reading DOs with/without treatment) = **70.5%**
clone 38: 1.00/1.21 = 82.6%

Ammonium thiocyanate protocol avidity:
Clone 10 (DOs):
Control: 1.12
Thiocyanate-treated samples:
2 M = 0.046; 1.5 M = 0,047; 1 M = 0.107; 0.75 M = 0.483; 0.5 M = 0.602; 0.375 M = 0.684
Avidity rate: **2.47**
Clone 38 (DOs):
Control: 1.22
Thiocyanate-treated samples:
2 M = 0.056; 1.5 M = 0.062; 1 M = 0.129; 0.75 M = 0.648; 0.5 M = 0.758; 0.375 M = 0.793
Avidity rate: **4.40**

It is seen that the avidity rates of clone 38 were higher than the ones of clone 10 in both assays. Furthermore, it is verified that, according to both protocols, it was necessary to use 50 times more antibody from clone 10 than from clone 38 in order to reach a DO close to 1.0.

6. Determination of association and dissociation constants of the monoclonal antibodies (clones 10 and 38 by surface plasmon resonance method [SPR] [BIAcore])

The results obtained by SPR method confirmed the preliminary results of antibody avidity, where clone 38 showed results higher than the ones of clone 10 again. According to the data seen in **Table II**, we see that clone 38 shows affinity 450 times higher than the clone 10 one.

This affinity is mainly due to its higher association rate, which is about 100 times higher than the clone 10 one. Due to the very high affinity of clone 38, **its measures present parameters close to the equipment detection threshold.** Yet, due to the care

in trial planning and conduction, we obtained an excellent adjustment for trial data using Langmuir's model, which indicates that the obtained data are reliable.

Figure 9 shows the interaction between recombinant PBP2a (antigen) and monoclonal antibodies clone 38 (Figure 9A) and clone 10 (Figure 9B). Smoky dashing curves represent SPR data in the concentrations according to the right key. All samples were analyzed in duplicate, and the 1:1 Langmuir's theoretical model for each curve is shown in black under each curve. Response units are represented in the vertical axis, and time is represented in the horizontal line in seconds. The closest lines referring to the horizontal axis represent the baseline for each sample (negative control).

Table II

Dissociation and interaction constants between antigen (PBP2a) and monoclonal antibodies clones 10 and 38.

	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
Clone 10	$4.42 \times 10^3 \pm 5.7$	$1.16 \times 10^{-4} \pm 5.63 \times 10^{-7}$	26.2
Clone 38	$5.45 \times 10^5 \pm 384$	$3.13 \times 10^{-5} \pm 2.11 \times 10^{-10}$	5.7×10^{-2}

7. Identification of complementary-determining regions (CDRs) of light and heavy chains of anti-PBP2a monoclonal antibody

After the mRNA extraction process in the hybridoma cells of the clone producing the used antibodies (clone 38), cDNA obtainment was performed and PCR reactions were performed with this material for different light and heavy chain alleles. The obtained materials were subjected to sequencing using the same starter sequences (defined by SEQ ID NO.: 18 to SEQ ID NO.: 39) used in PCR reactions. Light chain 391 and heavy chain 310 were identified in three distinct sequencings. Applying the Kabat's and Chotia's algorithms, we obtain the identification of light and heavy chain CDRs, which are the target of the attached claims.

We present the sequences of light and heavy chain CDRs below.

SEQ ID NO.: 6 - CDR 1 light chain amino acids.
RSSQSIGHSNGNTYLE
SEQ ID NO.: 7 - CDR 2 light chain amino acids.
KVSNRFS
SEQ ID NO.: 8 - CDR 3 light chain amino acids.
FQGSYVPLT
SEQ ID NO.: 9 - CDR 1 light chain DNA.
cgcagcagccagagcattggccatagcaacggcaacacctatctggaa
SEQ ID NO.: 10 - CDR 2 light chain DNA.
aaagtgagcaaccgctttagc
SEQ ID NO.: 11 - CDR 3 light chain DNA.
tttcagggcagctatgtgccgctgacc
SEQ ID NO.: 12 - CDR 1 heavy chain amino acids.
GFSITSSSSCWH

SEQ ID NO.: 13 - CDR 2 heavy chain amino acids.
RICYEGSISYSPSLKS
SEQ ID NO.: 14 - CDR 3 heavy chain amino acids.
ENHDWFFDV
SEQ ID NO.: 15 - CDR 1 heavy chain DNA.
ggctttagcattaccagcagcagcagctgctggcat
SEQ ID NO.: 16 - CDR 2 heavy chain DNA.
cgcatttgctatgaaggcagcattagctatagcccgagcctgaaaagc
SEQ ID NO.: 17 - CDR 3 heavy chain DNA.
gaaaaccatgattggttttttgatgtg

Complementary data

Further other assays were conducted, proceeding the invention development. These are reported below, through examples.

Example 3

A second study using CEB MRSA strain was conducted, following the same protocol described in Example 1, item 7.2, with adding of two pulses of agitation by vortex, of 15 seconds each one, after each washing; aiming to disaggregate *Staphylococcus aureus* agglomerates and to increase the quantity of PBP2a exposed to antibodies.

The markers FITC (fluorescein isothiocyanate) and PE (phycoerythrin) were tested, with control samples (i. pure bacterium, without contact with monoclonal antibody; and ii. pure bacterium plus FITC or PE marker) and the sample treated with monoclonal antibody and subjected to marking with PE or FITC being analyzed. The readings in FACsalibur device were performed in linear mode.

Figure 10 is a graph of analysis by flow cytometry of MRSA samples in presence of FITC-marked anti-PBP2a antibody. Curve (x) corresponds to non-marked sample, and curve (y) corresponds to marked sample.

The obtained results showed that about 22% of marked population was detected by the device, confirming the recognition of the target (PBP2a) present on the bacterial surface by anti-PBP2a antibodies (Figure 10).

Example 4

The inventors still surveyed the protection conferred by anti-PBP2a monoclonal antibody from methicillin-resistant *Staphylococcus aureus* against enterococcus. According to what was already previously mentioned here, the antibody recognizes proteins present in *Enterococcus* sp. strains, probably PBP5 - a transpeptidase with low affinity for beta-lactams, present in all enterococcus strains, with molecular weight of approximately 76 kDa (237 amino acids). This enzyme presents homology referring to PBP2a of MRSA, according to alignment (ClustalW) related below:

```
PBP5Efas        MERSNRNKKSSKNPLILGVSALVLIAAAVGGYYAYSQWQAKQELAEAKKTATTFLNVLSK 60
PBP5efam        -----KHGKNRTGAYIAG--AVILIAAAGGGYFYYQHYQETQAVEAGEKTVEQFVQALNK 53
PBP2a           ----------MKKIKIVPLILIVVVVGFGIYFYASKDKEINNTIDAIEDKNFKQVYKD 48
                              *     :::::..  * : *   :             * :. ..

PBP5Efas        QEFDKLPSVVQEASLKKNGYDTKSVVEKYQAIYSGIQAEGVKASDVQVKKAKDNQYTFTY 120
```



The alignment was performed with sequences corresponding to PBP5 from the enterococci *E. faecalis* (Efas) and *E. faecium* (Efam) with MRSA PBP2a. Marked sequences (bold - PBP5; underlined - PBP2a) correspond to the used PBP2a region to generate monoclonal antibodies. Amino acids corresponding to the enzyme active core are marked in italic.

Thus, *in vitro* protection assays, determination of lethal dose and LD_{50}, and *in vivo* assays (sublethal dose with renal quantification and survival assay with lethal dose)

with an enterococcus strain in murine model (Balb/C mice) were conducted, as previously conducted with MRSA. These results can be seen in the corresponding reports.

1.1. *In vitro* protection assays

The objective of this assay was to evaluate the *in vitro* protection conferred by the antibody against VRE enterococcus strain.

In vitro protection test (MIC), clone 38 purified monoclonal antibody (90/DA5/CB5/AA3 hib 77) against *Enterococcus f.* clinical strain (VRE), Richet laboratory.

Conditions:
Sample: purified from supernatant by HPLC, SelecSure MAB resin, dialysed, lyophilized.
Antibody quantification (Lowry's method): 3.5 mg/mL
Inoculum: VRE strain
Pre-inoculum: 1 VRE colony in 20 mL Lb and vancomycin (10 mg/mL), ON 37°C, 160 rpm
Inoculum: 400 mL of pre-inoculum in 20 mL Lb, 200-mL erlenmayer, 37°C, 160 rpm
DO_{600nm} reading after 7 hours: 0.7
Quantification: 5.5×10^8 bacteria/mL
Test conditions:
Inoculum: 5.5×10^5 bacteria
Antibody concentrations: 300, 400, 500, 600, and 700 mg of antibody
Culture medium: 1 mL of Luria broth
Cell culture plate, 24 cavities
Positive control: Luria broth + bacterial inoculum
Negative control: Luria broth
Incubation: 18 hours, 37°C

Figure 11 shows the result of the evaluation of the protection conferred by the antibody. In Figure 11, we have:

A. 300 mg of antibody
B. 400 mg of antibody
C. 500 mg of antibody
D. 600 mg of antibody
E. Negative control
F. Positive control
G. 700 mg of antibody

1.2. LD_{50} and lethal dose determination by intraperitoneal route - vancomycin-resistant *Enterococcus faecium*

Protocol:
Female 7-week Balb/C animals, mean weight of 20 grams

Day 01 - pre-inoculum: 1 VRE strain colony in 10 mL of Lb broth and vancomycin (10 mg/mL), 50-mL Falkow tube, growth ON 37°C, 160 rpm
Day 02 - inoculum: 1 mL of pre-inoculum in 50 mL of Lb broth/vancomycin (250-mL erlenmayer) - 4 vials, 37°C, 160 rpm, growth until OD_{600nm} = 0.80
centrifugation for 10 min, 4000 rpm, resuspension in PBS 1x sterile, OD 1.2
Quantification: 2.1 x 10^8 bacteria/mL
A. 60 microliters (1.5 x 10^7)
B. 300 microliters (6.5 x 10^7)
C. 900 microliters (reduced to 300 microliters/dose) (1.5 x 10^8)
D. 4.5 mL (reduced to 300 microliters/dose) (6.5 x 10^8)
E. 9.0 mL (1.2 x 10^9 bacteria) (reduced to 300 microliters/dose)
F. 45.0 mL (6.5 x 10^9 bacteria) reduced to 300 microliters/dose)

The animal observation was performed from day 02 to the tenth day of the assay.
The result is shown in Figure 12, and it is concluded that the lethal dose is 1.2 x 10^9 bacteria and LD_{50} is 6.5 x 10^8 bacteria.

Renal quantification of animals surviving in the seventh day:
A (1.5 x 10^7): no bacterial growth
B (6.5 x 10^7): no bacterial growth
C (1.5 x 10^8): 3100 bacteria
D (6.5 x 10^8): 2.8 x 10^4 bacteria

1.3. *In vivo* protection test - survival test - lethal dose, systemic infection by intraperitoneal route in murine model

The objective of this assay was to evaluate the efficacy of *in vivo* protection for anti-PBP2a monoclonal antibody against systemic infection with lethal dose, *Enterococcus faecium* (VRE) strain.

1. Antibody (purified from supernatant of the cell culture in medium with serum)
- Dialysed and lyophilized purified sample (HPLC SelecSure MAB), resuspended and filtered before use.
- Quantification (Lowry's method): 1.0 mg/mL
2. Murine model: female, 8-week Balb/C animals, weigh from 23 to 25 grams
3. Protocol:
group A (6 animals): 650 mg of antibody (350 mg + 300 mg)
group B (6 animals): control (saline administration)
4. Preparation of bacterial inoculum:
VRE strain:
pre-inoculum, day 01, 10 mL of BHI broth and vancomycin at 10 mg/mL ON, 37°C, 160 rpm
inoculum, day 03: 300 mL of pre-inoculum in 30 mL of BHI and vancomycin, DO_{600} of 1.31, centrifuged for 10 min, 4,000 rpm, resuspended in sterile PBS 0.5x, adjustment at OD = 1.10, dilutions and plates for quantification (2.0 x 10^8 bacteria/mL); inoculum: 12 mL, centrifugate resuspended in 300 mL, IP route (~2.2 x 10^9 bacteria)
Time schedule:
Day 01: IP inoculation of antibody (350 mg)

Day 02: IP inoculation of antibody (300 mg), systemic infection in the afternoon (IP, 250 mL of bacterial solution - 2.2 x 10^9 bacteria)
Day 02 to Day 13: animal observation.

The results are shown in Figure 13. Only 2 treated animals died (66.6% of protection). All control animals died in the second day.

1.4. *In vivo* protection test - systemic infection by intraperitoneal route in murine model with vancomycin-resistant *Enterococcus faecium*

The objective was to evaluate the *in vivo* protection efficacy of anti-PBP2a and PBP5 monoclonal antibody from enterococci against systemic infection, *E. faecium* (VRE) strain.

Test:
1. Antibody (purified from supernatant of the cell culture in medium with serum)
- Dialysed, lyophilized, and resuspended purified sample (AffiPrep ProteinA Biorad/HPLC SelecSure MAB).
- Quantification (Lowry's method): 1.5 mg/mL
2. Murine model: female, 8-week Balb/C animals, weigh from 19 to 23 grams
3. Protocol:
group A (4 animals): 500 micrograms of antibody (in 2 months, d01, d02)
group B (4 animals): non-protected control
4. Preparation of bacterial inoculum:
Iberian MRSA strain:
pre-inoculum, 10 mL of Lb broth ON, 37°C, 120 rpm
inoculum: 200 microliters of pre-inoculum in 20 mL of Lb, DO_{600} of 0.80, centrifuged for 10 min, 4,000 rpm, resuspended in sterile PBS 0.5x, adjustment at OD = 0.51, dilutions and plates for quantification (2.4 x 10^8 bacteria/mL); inoculum: 500 microliters, IP route (2.4 x 10^8 bacteria)
Time schedule:
Day 01: inoculation of 250 micrograms of antibody by intraperitoneal route
Day 02: inoculation of 250 micrograms of antibody by intraperitoneal route and systemic infection (intraperitoneal, 500 microliters of bacterial solution)
Day 06: euthanasia, bacterial quantification in kidneys

Based on the results above, we can highlight that:

***In vitro* protection assays** - determination of minimum inhibitory concentration:
A total of 700 micrograms of antibody is able to block the growth of 550,000 bacteria. These values are higher than the MIC obtained for MRSA strains, which were approximately 500 micrograms.

***In vivo* protection assays:**
***In vivo* protection test** - systemic infection by intraperitoneal route in murine model with sublethal dose of vancomycin-resistant *Enterococcus faecium*.

The animals received 500 micrograms of monoclonal antibody, intraperitoneal route (IP), and were subjected to systemic infection, IP route, with 2.4 x 10^8 bacteria. Four days after, they were subjected to euthanasia and excision of kidneys for bacterial quantification. Treated animals presented a mean of 87.5 bacteria/animal, while controls (non-treated infected animals) presented a mean of 211,000 bacteria/animal.

Survival test after lethal dose administration

The animals received 650 micrograms of monoclonal antibody (IP route) and were subjected to systemic infection (IP route) and daily observed for 10 days. Control (non-treated) animals died until the second day after the infection; two of the treated animals (6) died in the second day; the others remained alive until the end of the trial. Survival rate was 66.6%.

Therefore, once more we have confirmed that MRSA anti-PBPa monoclonal antibody showed to confer cross protection against enterococci. However, the doses needed for conferring protection were higher than the ones used against MRSA in similar conditions. This is probably due to the lower capacity of the antibody to recognize PBP5 with the same efficacy as PBP2a, which it was developed for.

Therefore, the current invention described here - anti-PBP2a monoclonal antibodies, able to specifically bind to PBP2a and homologous sequences - has applicability in infections caused by bacteria presenting this protein or similar substances (MRSA, MRSE, and *Enterococcus* spp., and any other pathogen that has a protein homologous to PBP2a).

It is important to emphasize that once these infections are a worldwide problem and the assays were conducted against the main known epidemic MRSA clones, the product of the current invention has application in any place where there are infections by this pathogen.

The documents relative to the state of the art of knowledge of the inventors, cited in the current descriptive report, are listed below.

1. KOPP, BJ. NIX DE, ARMSTRONG EP. Clinical and economic analysis of methicillin-resistant *Staphylococcus aureus* infections. The Annals of Pharmacotherapy; 38:1377-82. 2004.

2. BOYCE, JM. Are the epidemiology and microbiology of methicillin-resistant *Staphylococcus aureus* changing? JAMA; 279(8):623-4. 1998.

3. HUNT, C; DIONNE M, *et. al.* Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus* in Minnesota and North Dakota, 1997-1999. Morbidity and Mortality weekly report-CDC USA. 48(32):707-10. 1999.

4. O'BRIEN, FG; PEARMAN, JW; GRACEY, M; RILEY, TV. GRUBB, WB. Community strain of methicillin-resistant *Staphylococcus aureus* involved in a hospital outbreak. Journal of Clin Microbiol. 37(9):2858-62. 1999.

5. TENOVER, FC; LANCASTER, MV; HILL, BC; STEDWARD, CD; STOCKER, SA; HANCOCK, GA; *et. al.* Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. Journal of Clin Microbiol. 36(4):1020-27. 1998.

6. LUTZ, L; MATOS, SB; KUPLICH, N; MACHADO A; BARTH AL. Características laboratoriais de *Staphylococcus aureus* isolado de paciente que não respondeu ao

tratamento com vancomicina. Primeiro Encontro de Controle de Infecção Hospitalar do MERCOSUL. 1999.
7. COSGROVE, SE; CARROLL, KC; PERL, TM. *Staphylococcus aureus* with reduced susceptibility to vancomycin. Clin Infect Diseases. 39(4):539-45. 2004.
8. JARVIS, WR; SCHLOSSER JMA, CHINN RY, TWETTEN S, JACSON M. National prevalence of methicillin resistant *Staphylococcus aureus* in inpatients at US health care facilities, 2006. Am J Infect Control. 35(10):631-7. 2007.
9. YAMAUCHI, M. Japan struck by resistant *Staphylococcus aureus*. British Medical Journal. 306:740. 1993.
10. PANLILIO, AL; CULVER, DH; GAYNES, RP. Methicillin-resistant *Staphylococcus aureus* in US hospitals, 1975-1991. Infection Control and Hospital Epid. 13:582-86. 1992.
11. LOWRY, F. *Staphylococcus aureus* infections. New England Journal of Medicine. 339:520-32. 1998.
12. FARR, BM. Prevention and control of methicillin-resistant *Staphylococcus aureus* infections. Current Opinion Infectious Diseases. 17:317-22. 2004.
13. TIEMERSMA, EW; BRONZWA, ER; SLAM, *et. al.* Methicillin-resistant *Staphylococcus aureus* in Europe, 1999-2002. Emerging Infectious Diseases. 10:1627-34, 2004.
14. BERETTA, ALRZ; TRABASSO, P.; STUCCHI, RB; MORETTI, ML. Use of molecular epidemiology to monitor the nosocomial dissemination of methicillin-resistant *Staphylococcus aureus* in an University hospital from 1991-2001. Braz. Journal of Medical and Biological Research. 37:1345-51. 2004.
15. PANNUTI, CS; GRINBAUM, RS. An overview of nosocomial infection control in Brazil. Infection Control and Hospital Epidemiology. 16(3):170-74. 1995.
16. RESENDE, EM; COUTO, BRGM; STARLING, CEF; MÓDENA, CM. Prevalence of nosocomial infections in general hospitals in Belo Horizonte. Infection Control and Hospital Epidemiology. 19(11):872-76. 1998.
16a. Grundmann H., Aires de Souza M., Boyce J. and Tiemersma J. Emergence and resurgence of Methicillinn-resistant *Staphylococcus aureus* as a public trhreat health. Lancet. 2006. 368:874-85.
17. GUIGNARD, B; ENTENZA, JM; MOREILLON P. Beta-lactams against methicillin-resistant *Staphylococcus aureus*. Curr. Opin Pharmacol. 5(5):479-89. 2005.
18. SENNA, JP; ROTH, DM; OLIVEIRA, JS; MACHADO, DC, SANTOS DS. Protective immune response against methicillin resistant *Staphylococcus aureus* in a murine model using a DNA vaccine approach. Vaccine. 21:2661-66. 2003.
19. OHWADA, A; SEKIYA, M; HANAKI, H; ARAI, KK; HIRAMATSU, K, FUKUCHI,Y. DNA vaccination by mecA sequence evokes an antibacterial immune response against methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents and Chemother. 44:767-74. 1999.
20. DOMBROWSKI, JC; WINSTON, LG. Clinical failures of appropriately-treated methicillin-resistant Staphylococcus aureus infections. J. Infect. 57(2):110-5. 2008.
20bis. RYFFEL, C; TESCH, W; BICH-MACHIN, I.; REYNOLDS, PE; BARBERIS-MAINO, L; KAYSER, FH; BERGER-BACHI, B. Sequence comparison of *mec*A genes isolated from methicillin-resistant *S. aureus* and *S. epidermidis*. Gene. 94:137-38. 1990.

21. ROTH, DM; SENNA, JP; MACHADO, DC. Evaluation of the humoral immune response in BALB/c mice immunized with a naked DNA vaccine anti-methicillin-resistant *Staphylococcus aureus*. Genetics Molecular Research. 5(3):503-12. 2006.
22. YOKOYAMA, W. Production of monoclonal antibodies: induction of immune responses, p. 2.5.4-2.5.8. In: J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach and W. Strober (ed). Current Protocols in Immunology, vol. 1. Wiley and Sons, Hoboken, N.J. 1995.
23. REED, LJ AND MUENCH H. Am. J. Hyg. 27:493-97. 1938.
24. KABAT, EA; WU, TT; BILOFSKY, H; M REID-MULLER, PERRY, H. Sequence of proteins of immunological interest. National Institutes of Health, Bethesda. 1993.
25. CHOTIA, C; LESK, AM; TRAMONTANO, A; LEVITT, M; SMITH-GILL, PADLAN, EA; DAVIES, D; TULIP, WR. Conformations of immunoglobulin hypervariable regions. Nature, 342:877-883. 1989.
26. GOULD IM. The clinical significance of methicillin-resistant *Staphylococcus aureus*. J. Hospital Infections, 61(4):277-82. 2005.
27. ANDREMONT, A; TIBON-CORNILLOT, M. Le triomphe des bactéries, la fin des antibiotiques? Ed Max Milo, 1 ed, 2007.
28. Silverstein, Arthur M. *Paul Ehrlich's receptor immunology*. San Diego: Academic Press, 2002.
29. LIM, D; STRINADKA NC. Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant *Staphylococus aureus*. Nat Struct Biol. 9(11): 870-6. 2002.
30. PAPAKYIRIAKOU, H; VAZ, D; SIMOR, A; LOUIE M; MCGAVIN MJ. Molecular analysis of the accessory gene regulator (agr) locus and balance of virulence factor expression in epidemic methicillin-resistant *Staphylococcus aureus*. J Infect Dis. 181(3):2400-4. 2000.
31. TEIXEIRA, LA; RESENDE, CA; ORMONDE, LR; ROSENBAUM, R; FIGUEIREDO AM, DE LENCASTRE H, TOMASZ A. Geographic spread of epidemic multiresistant *Staphylococcus aureus* clone in Brazil. J Clin Microbiol. 33(9):2400-4.1995.
32. DA SILVA, COIMBRA MV; TEIXEIRA, LA; RAMOS, RL; PREDARI, SC; CASTELLO, L; FAMIGLIETTI A, VA, C; KLAN, L; FIGUEIREDO AM. Spread of the Brazilian epidemic clone of a multiresistant MRSA in two cities in Argentina. J Med Microbiol. 49(2):187-92. 2000.
33. SENNA, JP; PINTO, CA; MATEOS, S; QUINTANA, A; SANTOS DS. Spread of a dominant methicillin-resistant *Staphylococcus aureus* (MRSA) clone between Uruguayan and South Brazil Hospitals. J Hospital Infect. 53(2):15607. 2003.
34. MELTER, O; SANTOS SANCHES, I; SCHINDLER, J; AIRES DE SOUZA, M; KOVÁROVA, V; ZEMLICKOVÁ, H; DE LENCASTRE H. Methicillin-resistant *Staphylococcus aureus* clonal types in the Czech Republic. J Clin Microbiol, 37(9):2798-803. 1999.
35. DA SILVA COIMBRA MV, SILVA-CARVALHO MC; WISPLINGHOFF H; HALL GO; TALLENT S; WALLACE, S; EDMOND, MB; FIGUEIREDO, AM; WEINZEL, RP. Clonal spread of methicillin-resistant *Staphylococcus aureus* in a large geographic area of the United States. J Hosp Infect. 53(2):103-10. 2003.
36. NYGAARD, TK; DELEO, FR; VOYICH, JM. Community-associated methicillin-resistant *Staphylococcus aureus* skin infections: advances toward identifying the key virulence factors. Curr Op Infect Dis. 21(2):147-52. 2008.

37. DIEP, BA; CHAMBERS, HF; GRABER, CJ; SZUMOWSKI, JD; MILLER, LG; HAN, LL; CHEN, JH; LIN, F; *et. al.* Emergence of multidrug-resistant, community-associated, methicillin-resistant *Staphylococcus aureus* clone USA300 in men whohave sex with men. Ann Intern Med. 148(4):249-57. 2008.
38. REICHERT, JM; DEWITZ, MC. Anti-infective monoclonal antibodies: perils and promise of development. Nat Rev Drug Discov. 5(3):191-5. 2006.
39. GAO, J; STEWART, GC. Regulatory elements of the *Staphylococcus aureus* protein A (Spa) promoter. J Bacteriol. 186(12):3738-48. 2004.
40. GOFFIN, C; GHUYSEN JM. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and parologs. Microbiol Mol Biol Rev. 62(4):1079-93. 1998.
41. COURVALIN P. Vancomycin resistance in gram-positive cocci. Clin Infect Dis. 1;42 Suppl 1:S25-34. 2006.
42. BAILIE GR, NEAL D. Vancomycin ototoxicity and nephrotoxicity. Med Toxicol Adverse Drug Exp. 3:376-86. 1988.
43. SALGUERO E; PLAZA D; MARINO A; MORENO C; DELGADO G. Characterising vancomycin's immunotoxic profile using Swiss and CFW mice as na experimental model. Biomed Pharmacother. Sept 16. 2008.

SUMMARY

MONOCLONAL ANTIBODIES FOR PBP2-a PROTEIN AND HOMOLOGOUS SEQUENCES FOR TREATMENT OF INFECTIONS AND IMMUNODIAGNOSIS ON BACTERIA FROM PHYLUM *FIRMICUTES*

The current invention refers to monoclonal antibodies able to recognize and bind to PBP2a protein and other proteins presenting sequences homologous to PBP2a, including the pathogens methicillin-resistant *Staphylococcus aureus* - MRSA, coagulase-negative *Staphylococcus*, *Staphylococcus sciuri*, *Enterococcus* spp., and any other bacterium possessing PBP2a or sequences homologous to this protein.

The invention still refers to the use of monoclonal antibodies able to recognize and bind to PBP2a protein and other proteins presenting sequences homologous to PBP2a in a complementary immunodiagnosis for detection of resistance to beta-lactams.

CLAIMS

1. Isolated monoclonal antibody that binds itself to PBP2a protein from methicillin-resistant bacteria (MRSA), characterized by comprising a variable chain region that comprises an amino acid sequence, according to what is selected from groups SEQ ID NO.: 6, SEQ ID NO.: 7, SEQ ID NO.: 8, SEQ ID NO.: 12, SEQ ID NO.: 13, and SEQ ID NO.: 14 or corresponding amino acid sequences at least 80% identical to them.

2. Isolated monoclonal antibody that binds itself to PBP2a protein from methicillin-resistant bacteria (MRSA), characterized by comprising a variable chain region that comprises an DNA sequence, according to what is selected from groups SEQ ID NO.: 9, SEQ ID NO.: 10, SEQ ID NO.: 11, SEQ ID NO.: 15, SEQ ID NO.: 16, and SEQ ID NO.: 17 or corresponding DNA sequences at least 80% identical to them.

3. Isolated monoclonal antibody that binds itself to PBP5 protein from *Enterococcus* spp., characterized by comprising a variable chain region that comprises an amino acid sequence, according to what is selected from groups SEQ ID NO.: 6, SEQ ID NO.: 7, SEQ ID NO.: 8, SEQ ID NO.: 12, SEQ ID NO.: 13, and SEQ ID NO.: 14 or corresponding amino acid sequences at least 80% identical to them.

4. Isolated monoclonal antibody that binds itself to PBP5 protein from *Enterococcus* spp., **characterized by** comprising a variable chain region that comprises an DNA sequence, according to what is selected from groups SEQ ID NO.: 9, SEQ ID NO.: 10, SEQ ID NO.: 11, SEQ ID NO.: 15, SEQ ID NO.: 16, and SEQ ID NO.: 17 or corresponding DNA sequences at least 80% identical to them.

5. Method for diagnosis of resistance to beta-lactam antimicrobials, **characterized by** comprising the addition of an isolated monoclonal antibody, according to what is defined in one of claims 1 or 2, pure or in association to other substances, for bacterial samples suspected of containing PBP2a or proteins homologous to it.

6. Pharmaceutical composition for treatment or prevention of infection by bacteria possessing PBP2a or proteins homologous to it, **characterized by** comprising a quantity of the isolated monoclonal antibody according to what is defined in claims 1 or 2 and a pharmaceutical vehicle, either carrier or excipient.

7. Method for treating or preventing an infection by a bacterium possessing PBP2a or proteins homologous to it, **characterized by** comprising the administration of a quantity of the isolated monoclonal antibody to a human or animal patient, according to what is defined in claims 1 or 2.

8. Antibody according to claim 1, **characterized by** a variable heavy chain possessing a CDR1 region that includes the sequence **GFSITSSSSCWH** (SEQ ID NO.: 12) (CDR1 VH) and the corresponding nucleic acid sequence, with the possible degenerations.

9. Antibody according to claim 1, **characterized by** a variable heavy chain possessing a CDR2 region that includes the sequence **RICYEGSISYSPSLKS** (SEQ ID NO.: 13) (CDR2 VH) and the corresponding nucleic acid sequence, with the possible degenerations.

10. Antibody according to claim 1, **characterized by** a variable heavy chain possessing a CDR3 region that includes the sequence **ENHDWFFDV** (SEQ ID NO.: 14) (CDR3 VH) and the corresponding nucleic acid sequence, with the possible degenerations.

11. Antibody according to claim 1, **characterized by** a variable light chain possessing a CDR1 region that includes the sequence **RSSQSIGHSNGNTYLE** (SEQ

ID NO.: 6) (CDR1 VL) and the corresponding nucleic acid sequence, with the possible degenerations.

12. Antibody according to claim 1, **characterized by** a variable light chain possessing a CDR2 region that includes the sequence **KVSNRFS** (SEQ ID NO.: 7) (CDR2 VL) and the corresponding nucleic acid sequence, with the possible degenerations.

13. Antibody according to claim 1, **characterized by** a variable light chain possessing a CDR3 region that includes the sequence **FQGSYVPLT** (SEQ ID NO.: 8) (CDR3 VL) and the corresponding nucleic acid sequence, with the possible degenerations.

14. Antibody according to claim 2, **characterized by** a variable light chain possessing a CDR1 region that includes the sequence SEQ ID NO.: 9 and the corresponding nucleotide sequence, with the possible degenerations.

15. Antibody according to claim 2, **characterized by** a variable light chain possessing a CDR2 region that includes the sequence SEQ ID NO.: 10 and the corresponding nucleotide sequence, with the possible degenerations.

16. Antibody according to claim 2, **characterized by** a variable light chain possessing a CDR3 region that includes the sequence SEQ ID NO.: 11 and the corresponding nucleotide sequence, with the possible degenerations.

17. Antibody according to claim 2, **characterized by** a variable heavy chain possessing a CDR1 region that includes the sequence SEQ ID NO.: 15 and the corresponding nucleotide sequence, with the possible degenerations.

18. Antibody according to claim 2, **characterized by** a variable heavy chain possessing a CDR2 region that includes the sequence SEQ ID NO.: 16 and the corresponding nucleotide sequence, with the possible degenerations.

19. Antibody according to claim 3, **characterized by** a variable heavy chain possessing a CDR3 region that includes the sequence SEQ ID NO.: 16 and the corresponding nucleotide sequence, with the possible degenerations.