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Construção de quimeras recombinantes contendo antígenos de *Bartonella henselae*

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“O maior bem que podemos fazer a um homem é levá-lo à Verdade.”
Santo Tomás de Aquino

Resumo

GONÇALVES, Jênifer Malheiros. **Construção de quimeras recombinantes contendo antígenos de *Bartonella henselae*.** 2020. 98f. Tese (Doutorado em Ciências) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Bartonella henselae é uma bactéria Gram-negativa e intracelular facultativa, onde os gatos são seu reservatório primário e os seres humanos seus hospedeiros acidentais. *B. henselae* é causadora da zoonose conhecida como Doença da Arranhadura do Gato (*Cat Scratch Disease* - CSD). O diagnóstico da CSD ainda é um desafio, pois as técnicas convencionais de detecção de antígenos ou anticorpos têm demonstrado limitações. O diagnóstico padrão-ouro da CSD é a Imunofluorescência, com boa sensibilidade, entretanto, com possibilidade de reações cruzadas com outras espécies. Diante disto, as ferramentas de bioinformática têm sido utilizadas em todo o mundo, otimizando a busca por novas alternativas de diagnóstico. Assim, o objetivo do presente trabalho foi identificar, através de análises de imuno-bioinformática, epítopos de *B. henselae*, e construir quimeras recombinantes multi-epítopo para uso como insumos biotecnológicos. Os sete antígenos mais citados em artigos científicos (GroEL, 17kDa, P26, BadA, Pap31, OMP89 e OMP43) foram escolhidos para a fase de identificação e seleção dos epítopos. Os epítopos de células B foram selecionados usando dois preditores (BepiPred e Iedb). O estudo tridimensional das proteínas, bem como análises de qualidade estrutural, análises físico-químicas e de antigenicidade foram feitos através do servidor I-TASSER, QMEAN6 e do servidor Protoparam, respectivamente. A análises das estruturas secundárias do mRNA foram performadas para verificar a estabilidade da tradução das proteínas através do software RNAfold. A seguir, o desenho *in silico* das quimeras foi feito e as mesmas enviadas para síntese química. Três quimeras recombinantes (rC) foram constituídas, sendo elas compostas por epítopos presentes nas proteínas GroEL, 17kDa e P26 (rC1); BadA, Pap31 e GroEL (rC2) e P26, OMP89 e OMP43 (rC3). As quimeras recombinantes (rC) foram clonadas em vetor de expressão em *Escherichia coli*, pAE. A antigenicidade das proteínas foi verificada através de *Western Blot* (WB) com soro de humano naturalmente infectado por *B. henselae*. Todas as proteínas quiméricas foram expressas com sucesso, de forma insolúvel, e com massa molecular esperada de 36 kDa (rC1), 34 kDa (rC2) e 38 kDa (rC3). As análises de qualidade demonstraram que

a rC2 foi a construção ligeiramente melhor que as demais. As análises das estruturas secundárias do mRNA revelaram que estes tinham estabilidade suficiente para traduzir efetivamente as proteínas. Todas as quimeras mostraram-se antigênicas através do WB. As quimeras recombinantes produzidas têm potencial para uso biotecnológico no desenvolvimento de testes de diagnóstico.

Palavras-chave: Proteína recombinante, CSD, doença da arranhadura do gato, *in silico;* diagnóstico.

Abstract

GONÇALVES, Jênifer Malheiros. **Construction of recombinant chimeras containing *Bartonella henselae* antigens.** 2020. 98f. Tese (Doutorado em Ciências) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Bartonella henselae is a facultative gram-negative and intracellular bacterium, where cats are its primary reservoir and humans are accidental hosts. *B. henselae* is considered one of the most important medical species, causing the zoonosis known as Cat Scratch Disease (CSD). The diagnosis of CSD is still a challenge since conventional techniques for detecting antigens or antibodies have demonstrated limitations. The gold standard diagnosis of CSD is IFI, with good sensitivity, however, with the possibility of cross-reactions with other species. Given this, bioinformatics tools have been used worldwide, optimizing the search for new diagnostic alternatives. Thus, the objective of the present work was to identify, through immuno-bioinformatics analyzes, epitopes of *B. henselae*, and to build multi-epitope recombinant chimeras for use as biotechnological inputs. The seven most cited antigens in scientific articles (GroEL, 17kDa, P26, BadA, Pap31, OMP89 and OMP43) were chosen for the epitope identification and selection phase. B cell epitopes were selected using two predictors (BepiPred and Iedb). The three-dimensional study of proteins, as well as structural quality analyzes, physical-chemical and antigenicity analyzes were performed using the I-TASSER, QMEAN6 and Protoparam servers, respectively. The analysis of the secondary structures of the mRNA were performed to verify the stability of the translation of proteins using the RNAfold software. Next, the *in silico* drawing of the chimeras was done and sent to chemical synthesis. Three recombinant chimeras (rC) were formed, being composed of epitopes present in the GroEL, 17kDa and P26 (rC1) proteins; BadA, Pap31 and GroEL (rC2) and P26, OMP89 and OMP43 (rC3). Recombinant chimeras (rC) were cloned into an expression vector in Escherichia coli, pAE. The antigenicity of the proteins was verified through Western Blot (WB) with human serum naturally infected by *B. henselae*. All chimeric proteins were successfully expressed, insoluble, and with an expected molecular mass of 37 kDa (rC1), 35 kDa (rC2) and 38 kDa (rC3). Quality analyzes showed that rC2 was a slightly better construction than the others. Analyzes of secondary mRNA structures revealed that they had sufficient stability to effectively translate proteins. All chimeras were shown to

be antigenic through the WB. The chimeras produced have potential for use in diagnostic tests.

Palavras-chave: Recombinant protein; CSD; Cat Scratch Disease; *in silico*; diagnosis.

Sumário

1 INTRODUÇÃO GERAL

Bartonella henselae é uma bactéria Gram-negativa, intracelular facultativa, que tem os gatos como seu reservatório primário, enquanto os seres humanos são considerados seus hospedeiros acidentais (GIL et al., 2013). Dentro do gênero, *B. henselae* é considerada a espécie de maior importância médica, pois infecta gatos e seres humanos, sendo considerada uma zoonose (BREITSCHWERDT, 2017). B. henselae, e outras espécies do gênero, representam um dos maiores desafios contemporâneos dentro do conteito "*One Health*" (BREITSCHWERDT, 2017) - que é um conceito proposto mundialmente -, e visa uma integração maior entre a medicina humana e veterinária (LANNINO et al., 2018). Neste contexto, as bartoneloses oferecem uma oportunidade para demonstrar os benefícios sociais de uma abordagem "*One Health*" para prevenir e aumentar o entendimento médico destas doenças infecciosas emergentes.

A transmissão de *B. henselae* para humanos ocorre através de mordidas ou arranhaduras de gatos infestados com pulgas, ou ainda, diretamente através de sangue contaminado (REGIER et al., 2016). Em humanos, *B. henselae* é o agente etiológico da Doença da Arranhadura do Gato (*Cat Scratch Disease* – CSD), que é uma infecção frequentemente autolimitante em indivíduos imunocompetentes, porém, pode ser fatal em indivíduos imunocomprometidos (REGIER et al., 2016). No Brasil, estudos epidemiológicos têm demonstrado a presença das espécies de *Bartonella,* tanto em humanos quanto em animais como gatos, cães, ratos e animais silvestres (COSTA et al., 2005; DINIZ et al., 2007; CRISSIUMA et al., 2011; BORTOLI et al., 2012; BRAGA et al., 2012; LAMAS et al., 2013; MICELI et al., 2013; costa ET AL., 2014; FAVACHO et al., 2014; KITADA et al., 2014; ANDRÉ et al., 2015; PITASSI et al., 2015; VIEIRA-DAMIANI et al., 2015; MALHEIROS et al., 2016; FONTALVO et al., 2017; SILVA et al., 2017; DRUMMOND et al., 2018; DRUMMOND et al., 2019). Além disso, estudos demonstraram, pela primeira vez, a presença de bacteremia por *Bartonella* spp. em doadores de sangue assintomáticos (PITASSI et al., 2015; VIEIRA-DAMIANI et al., 2015).

O diagnóstico da CSD ainda é um desafio, o que pode, de certa forma, contribuir para uma epidemiologia incompleta e, o mais importante, retardar o tratamento desta enfermidade, visto que não existe nenhuma vacina até o momento para prevenção. Segundo Breitschwerdt (2017), as técnicas convencionais de

isolamento bacteriano, ELISA (*Enzyme Linked Immuno Sorbent Assay*), *Western Blotting* ou Imunofluorescência para detecção de anticorpos e reação em cadeia da polimerase (*Polymerase Chain Reaction* - PCR) para amplificação de DNA têm demonstrado limitações.

A imunofluorescência indireta (IFI) é considerada padrão-ouro no diagnóstico da CSD, sendo um teste sorológico que apresenta boa sensibilidade, entretanto, é laborioso e tem possibilidade de reações cruzadas com *Chlamydia pneumoniae* e *Coxiella burnetti,* por exemplo (WAGNER et al., 2008). Desta forma, abre-se a possibilidade para o uso de proteínas recombinantes purificadas a fim de melhorar a sensibilidade e especificidade destes testes (FERRARA et al., 2014). Além disso, é importante que as proteínas sejam específicas, presentes apenas nos micro-organismos de interesse e ausentes nos demais. Neste sentido, estudos recentes têm utilizado proteínas de *B. henselae*, de forma recombinante, em ensaios sorológicos, principalmente ELISA, com resultados promissores (KABEYA et al., 2003; LITWIN et al., 2004; WAGNER et al., 2008; WERNER et al., 2008; ANGKASEKWINAI et al., 2014; FERRARA et al., 2014; JOST et al., 2018).

O desenvolvimento de insumos para uso em diagnóstico sorológico contra *B. henselae* é de considerável interesse, tanto na medicina humana quanto na veterinária, contribuindo para o correto tratamento das enfermidades causadas por este agente, bem como, para o real conhecimento de sua epidemiologia. Com base nisto, a proposta desta tese é utilizar a análise "*in silico"* para identificar epítopos presentes em proteínas antigênicas de *B. henselae*, e utilizá-los na construção de quimeras recombinantes para uso em diagnóstico.

A tese está apresentada na forma de manuscritos científicos. O manuscrito 1 é uma revisão bibliográfica sobre *B. henselae*, com ênfase no diagnóstico e seus desafios. Esse trabalho está formatado para o periódico *Acta Tropica*. O manuscrito 2 descreve a produção de proteínas quiméricas recombinantes contendo epítopos antigênicos de antígenos de *B. henselae,* selecionados por análises *in silico*. Esse trabalho foi submetido para publicação no periódico *Current Microbiology,* no ano de 2020. Também foi solicitado junto ao Instituto Nacional de Propriedade Intelectual (INPI) o depósito de uma patente, tendo como número de protocolo BR 1 027574 0.

2 REVISÃO DE LITERATURA

2.1 Manuscrito 1 - Challenges and new perspectives in diagnostic of Cat Scratch Disease

Manuscrito de revisão formatado de acordo com as normas do periódico *Acta Tropica*

Challenges and new perspectives in diagnostic of Cat Scratch Disease

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ABSTRACT

Bartonella henselae is the causative of zoonosis known as Cat Scratch Disease, which has cats as its primary reservoir and human as its accidental host. The diagnosis of bartonellosis is still a challenge, a fact that may contribute to an incomplete epidemiology and delay its treatment, since there is no vaccine so far. Conventional bacterial isolation techniques, ELISA (Enzyme Linked Immuno Sorbent Assay), Western Blotting or Indirect Immunofluorescence (IFI) for antibody detection and polymerase chain reaction (PCR) for DNA amplification have demonstrated limitations. In serological tests, the diagnosis is made by IF (gold standard), with good sensitivity, however, with the possibility of cross reactions with *Chlamydia pneumoniae* and *Coxiella burnetti,* for exemple. The search for new antigen candidates is necessary so

that they can be used in new serological tests. Thus, the proposal of this review is to discuss the main aspects related to *B. henselae*, especially addressing the existing diagnostic challenges, and their new perspectives.

Keywords: *Bartonella henselae*; diagnosis; recombinant antigens; CSD.

1. Introduction

Bartonella henselae belongs to the Rhizobiales family, is a α-Gram-negative, facultative intracellular growth proteobacteria and can infect the erythrocytes and endothelial cells of its hosts (GIL et al., 2013). Cats (*Felis catus*) are considered the primary reservoirs for *B. henselae*, however, dogs (*Canis familiaris*) have also been targeted as reservoirs, while humans are considered accidental hosts (BREITSCHWERDT, 2017). Within the genus *Bartonella*, currently 36 species are described, and, of these, there are around 22 species related to diseases in mammals (PENNISI et al., 2013; BREITSCHWERDT, 2017). However, *B. henselae* is considered one of the most important medical species because it infects cats and humans and is an emerging zoonosis (BREITSCHWERDT, 2017). The other species of medical importance are *B. bacilliformis* (Trench Fever) and *B. quintana* (Carrion's Disease).

The genre *Bartonella* and bartoneloses represent one of the greatest contemporary challenges within the "One Health" concept (BREITSCHWERDT, 2017). This worldwide concept proposes a much closer integration of human and veterinary medicine (LANNINO et al., 2018). In this context, bartoneloses offer an opportunity to demonstrate the social benefits of a "One Health" approach to prevent and increase medical understanding of this emerging infectious disease.

2. Transmission

Studies indicate that the transmission of *B. henselae* to cats occurs mainly by fleas (*Ctenocephalides felis*). This transmission may occur through direct contact or through stool-infected wounds of these fleas, as well as through ticks, which have also been suggested as potential vectors for this bacterium (REGIER et al., 2016; BREITSCHWERDT, 2017). Furthermore, research indicates that the presence of arthropod vectors is essential for the transmission of *B. henselae* among cats, because when they are healthy, but share the same environment with infected cats are free of ectoparasites, they do not become seropositive (REGIER et al., 2016).

In infected cats, *B. henselae* can survive inside erythrocytes, which when ingested by fleas remain in their intestines. Later, flea feces with *B. henselae* may be deposited on the skin of cats, and the cats, when they scratch themselves, harbour the bacteria in their claws (PENNISI et al., 2013). In addition, *B. henselae* may persist viable for at least nine days in flea faeces (BREITSCHWERDT, 2017). In addition to these forms of transmission to cats, blood transfusions also appear to pose a risk as cats have been experimentally infected with *B. henselae* through intramuscular and intravenous blood inoculation from infected cats (ABBOTT et al., 1997). In another study, transmission was proven through rats (SILVA et al., 2016).

The transmission of *B. henselae* to humans occurs through bites or scratches from flea-infested cats, which eliminate the bacteria in their feces, or directly through contaminated blood (REGIER et al., 2016). In addition to cats (domestic and wild), raccoons, mongooses and other animals can serve as a source of *B. henselae* infection for humans and other animals such as domestic dogs. In addition to fleas and ticks, mites and spiders can also contribute to its transmission (BREITSCHWERDT, 2017).

Interestingly, recent Brazilian studies have demonstrated the presence of bacteremia by *Bartonella* spp. in asymptomatic blood donors, reinforcing the need to assess the transmission of *Bartonella* by blood (PITASSI et al., 2015; VIEIRA-DAMIANI et al., 2015).

3. Clinical Signs

Cats infected with *B. henselae* are often clinically asymptomatic, although they suffer from long-term recurrent bacteremia (REGIER et al., 2016). Naturally infected cats may show mild clinical signs, which are often not perceived by owners and veterinarians. Fever, uveitis, endocarditis and lymphadenomegaly have already been reported. However, most clinical signs observed are from experimental studies of infection in cats, being moderate and varying according to the strain of *B. henselae* used (GUPTILL, 2010).

In humans, *B. henselae* is the etiologic agent of Cat Scratch Disease (CSD), which is a frequently self-limiting infection in immunocompetent individuals (REGIER et al., 2016); and has regional lymphadenopathy as its main symptom (GIL et al., 2013), which may be suspected of CSD, especially when there is a history of exposure to kittens and adult cats (LANNINO et al., 2018). CSD is often diagnosed in children but may also affect adults. Other symptoms may involve fever, headache, skin lesions and mucous membranes near the inoculation site and splenomegaly. Endocarditis, oculoglandular involvement (Parinaud's Syndrome), encephalopathies, neuroretinitis and osteomyelitis are described as complications of infection. In immunocompromised individuals, chronic infections can occur, leading to angioproliferative diseases such as bacillary angiomatosis and hepatic peliosis, which can be fatal if not treated (REGIER et al., 2016).

4. Epidemiology

Bartonella species have a worldwide distribution, being more pronounced in areas where conditions are more favorable for arthropod vectors, mainly fleas (PENNISI et al., 2013). In flea-endemic areas, seroprevalence levels of *Bartonella* spp. in cats can be higher than 90%, and bacteremia levels higher than 50%. Initially, *B. henselae* was isolated from an HIV-positive individual, and subsequently from cats around the world. Currently, *B. henselae* bacteremia has been reported in many different species, such as cows, horses, marine mammals, small land mammals and sea turtles, making the epidemiology of this species more complex than initially described (BREITSCHWERDT, 2017).

In the United States, a review of CSD cases from 2005 to 2013 showed that the incidence of the disease was higher in individuals living in the South and in children aged 5-9 years. The authors also estimate that 12,000 individuals are diagnosed with CSD each year, and that 500 of these are hospitalized. Furthermore, during the study, the authors identified 13,273 individuals diagnosed with CSD (NELSON et al., 2016).

In Korea, 300 healthy individuals composed a survey for *B. henselae* through IFI. Seroprevalence in those individuals who had been bred with cats was 22.2%, while in those who had not been bred seroprevalence was 13.7%. On the other hand, individuals who had cats as pets, seroprevalence was 9.8%, and in those who had not been bred and had no cats, seroprevalence was 2.0% (KWON et al., 2017).

In Brazil, some epidemiological studies have demonstrated the presence of *Bartonella* species (Table 1). The findings of *Bartonella* spp. range from rats (COSTA et al., 2014), cats (ANDRÉ et al., 2015; BORTOLI et al., 2012; BRAGA et al., 2012; CRISSIUMA et al., 2011; DRUMMOND et al., 2018; FONTALVO et al., 2017; KITADA et al., 2014; MALHEIROS et al., 2016; MICELI et al., 2013; SILVA et al., 2017;

STTAGEMEIER et al., 2014), dogs (DINIZ et al., 2007; FONTALVO et al., 2017) to humans (COSTA et al., 2005; DRUMMOND et al., 2019; FAVACHO et al., 2014; LAMAS et al. 2013; PITASSI et al., 2015; VIEIRA-DAMIANI et al., 2015) and come from the most diverse regions of Brazil. Seroprevalences range from 3.5 - 24.7% in dogs, 15 - 56.6% in cats, and 3.2 - 23.5% in humans. By molecular methods, the prevalences found vary from 0.5% in dogs, 2.2 - 90.2% in cats, and in humans from 3.2 – 26,5%. Different methods have been applied for the detection, such as PCR, IFI, ELISA and culture.

5. Molecular Biology

The complete genome of *Bartonella henselae* have 1,931,047 base pairs (ALSMARK et al., 2004). The 16S rDNA sequence, which was considered to be one of the most useful and informative tools for the identification and phylogenetic studies of bacteria, was the first gene to be studied but has failed to establish a reliable phylogeny of *Bartonella* species (ZEAITER et al., 2002). Genotypic analyses of *B. henselae* isolates using a variety of different pangenomic or locus-specific typing approaches have identified a number of genetic groupings. These analyses have been applied not only to epidemiological investigations of human *B. henselae* infections but also to broader surveys of human and feline isolate collections. Such surveys have revealed limited diversity among human-infecting isolates and even some type-specific differences in virulence but, yet no direct evidence for any particular hypervirulent genotypes. Several *B. henselae* genes and genetic loci have been subjected to comparative analysis for typing purposes, including the 16S and 23S ribosomal DNA (rDNA), 16S-23S rDNA intergenic spacer region, and fragments of protein-encoding genes such as *gltA*, *ftsZ*, and *pap31* (IREDELL et al., 2003).

Examination of 16S rRNA-encoding gene sequences has revealed that *B. henselae* isolates possess one of two different sequence types, with strains being designated as type I (Houston) type II (Marseile) on this basis. These types, or genotypes are more detected in some specific regions, like the genotype I was predominant in cats from Asia, and genotype II was detected most often in cats from USA, Europe and Australia (GIL et al., 2013). Furthermore, cats are most often infected with one or the other type, but some cats are co-infected with both types, and both types can be transmitted to humans from pets (VIGIL et al., 2010), however, the genotype I seemed to be more frequently associated with infections in humans (GIL et al., 2013). But although the genome sequence of *B. henselae* was published, not too much is known about the mechanisms underlying the virulence of this humanpathogenic bacterium (EBERHARDT et al., 2009).

In the study of Eberhardt et al. (2009), they identified 431 protein spots representing 191 different proteins. These proteins comprise predominantly cytosolic proteins and to a lesser degree periplasmic, cytoplasmic membrane and outer membrane proteins (OMPs).

Studies point to OMPs of *Bartonella* as being important, where they are essential for attachment, invasion, and survival within host cells, as seen in other Gram-negative human pathogenic bacteria. Since OMPs are the interface between the bacterium and the host cells, the outer membrane (OM) and individual OMPs are targeted in developments of diagnostic markers and vaccines against many bacterial diseases (LI et al., 2011). BadA is crucial for the adhesion of *B. henselae* to host cells and extracellular matrix proteins and is also important for the induction of an HIF-1- ediated angiogenic gene programming in infected host cells. Other OMPs of *B. henselae* are less intensively investigated although these surface-exposed determinants might be

highly important pathogenicity factors. Initially, nine *B. henselae* OMPs were identified of which the 43 kDa OMP was shown to adhere to endothelial cells. Hemin binding was reported for a 31 kDa protein (Pap31) and the 43 kDa OMP (EBERHARDT et al., 2009).

6. Diagnosis

An accurate microbiological diagnosis of CSD can be a great challenge, especially in patients with chronic and long-term infection. Conventional bacterial isolation techniques, Enzyme Linked Immuno Sorbent Assay (ELISA), Western Blotting (WB) or Immunofluorescence (IF) for antibody detection and polymerase chain reaction (PCR) for DNA amplification have demonstrated limitations (BREITSCHWERDT, 2017).

The diagnosis of *Bartonella* spp. infection has been considered a challenging task due to the difficulty to isolate these bacteria in vitro, requiring specific conditions. These characteristics limited the detection of infected animals and the identification of the species involved since they are inert to most classical biochemical assays. Molecular detection assays (PCR) were rapidly adopted to improve their sensitivity and facilitate the identification (GUTIÉRREZ et al., 2017). Below, we will address the main tools used in the diagnosis of bartonellosis.

6.1 Culture

The microbiological diagnosis of all *Bartonella* infections should be ideally confirmed by culturing the organism from blood, cerebrospinal fluid, joint fluid or aseptically obtained tissues (lymph node, spleen, heart valve) or by PCR amplifying *Bartonella* DNA directly from diseased tissues. However, the isolation of these same

Bartonella spp. from dog, horse or human blood samples using an identical isolation approach is very insensitive (BREITSCHWERDT, 2017).

Bartonellae are fastidious bacteria characterized by slow-growing rates. While the isolation of *Bartonella* species from natural reservoir hosts is relatively easy, it is more difficult from incidental hosts. The slow growth and the need for special growing conditions combined with low bacteremia make the laboratory isolation of *Bartonella* spp. from opportunistic hosts such as humans a great challenge (GUTIÉRREZ et al., 2017).

Several culture media have been used for the isolation of *Bartonella*. Columbia agar, *Brucella* agar, Brain Heart Infusion broth (BHI) and Trypticase soy-based agars, all supplemented with rabbit or sheep blood 5%, and chocolate agar (GUTIÉRREZ et al., 2017). However, the use of blood from animals for the isolation of *Bartonella* may be impaired due to contamination of this material.

Studies have reported the importance of an enrichment of blood cultures and a subcultivation to improve diagnosis (DRUMMOND et al., 2018). In this context, "*Bartonella* alpha-Proteobacteria growth medium" (BAPGM) is a new, chemically modified medium based on liquid culture containing insect cells that allow the growth of *Bartonella* species. This medium combines the enrichment of the culture of a clinical specimen in the liquid medium for a minimum period of 7 days and must be followed by a PCR assay (BREITSCHWERDT, 2017). However, BAPGM is not a selective medium for *Bartonel*la, and in cases of co-infections with other bacteria, primary isolation may be difficult (GUTIÉRREZ et al., 2017).

6.2 Serology

As with other infectious diseases, seroconversion of at least a fourfold increase in antibody titer over a period of 2 to 3 weeks can be used for the confirmation of acute cases of bartonellosis. However, most dogs, some cats, and many humans with bacteremia for *Bartonella* do not have antibodies production (BREITSCHWERDT, 2017). Thus, antibodies detection in the diagnosis of *B. henselae* is considered difficult and impaired by the low sensitivity and specificity of the assays used in the laboratory routine (OTSUYAMA et al., 2016). Serological tests can be used to support a clinical diagnosis, but the presence of antibodies can be used only to infer exposure to the bacterium (BREITSCHWERDT, 2017).

6.2.1 IFI

IFI test is used to detect antibodies to *B. henselae* in serum. Although IFI is the most widely used technique and is considered “golden standard”, it is more laborious and with less objective interpretation than the ELISA technique. Evaluations of serological tests have shown variations in sensitivity and specificity levels depending on population, definition of CSD and materials and techniques used. Furthermore, comparison of serological tests from different laboratories is also complicated by the lack of standardization (VERMEULEN et al., 2007).

Antibodies detection by some IFI with commercial kit uses whole cell antigens co-cultivated in Vero cells (Focus Diagnostics®, Fuller Laboratories®); this technique has been modified since its description in 1992 by Regnery and collaborators. The use of such cells provides substrate to the bacteria, which can adhere; helps to prevent self-agglutination among the bacteria; and provides a background that assists the focus on less fluorescent samples (DALTON et al., 1995). However, the study of Vermeulen et. al (2007) comparing the sensitivity of the commercial kit (Focus

Technologies) with an in-house IFI, obtained a sensitivity of only 6%, differently from the sensitivity reported in the brochure containing the product information, of 41.5%.

Currently, the internationally accepted IgG antibody titers are the clearly positive titers from 1:200/256/320, depending on the dilution scheme. For IgM there are no well-defined cut-off point values (JOST et al., 2018). IFI has good sensitivity, but these tests are considered expensive and with the possibility of cross reactions (FERRARA et al., 2014). In this context, it is proven that cross-reactions are greater than 50%, with many pathogens, such as *Chlamydia pneumoniae* and *Coxiella burnetti* (WAGNER et al., 2008).

6.2.2 ELISA

An ELISA allows the automated handling of many serum samples; in addition, it allows epidemiological studies to be conducted; and further, an ELISA represents a significant step in improving the serological diagnosis of infections caused by *B. henselae* (JOST et al., 2018). In this method, whole-cell antigens have also been used, but besides low sensitivity, they have little specificity (JOST et al., 2018; FERRARA et al., 2014). The detection of anti-*B. henselae* IgM may be more indicative of an active *B. henselae* infection. The performances of the IgM ELISA described so far have proven to be better than those of most IgG assays, but the sensitivities need to be increased (BERGMANS et al., 1997).

In the study of Bergmans et al. (1997), they evaluated the diagnostic performance of *B. henselae*-based IgG and IgM IFI and IgG and IgM ELISA with sera from patients with clinical CSD. The study group consisted of 45 patients suspected of suffering from CSD by fulfilling one or more of the classical criteria. The specificities of the immunoassays were set at >95% by analysis of sera from 60 healthy blood donors.

It is shown that the sensitivities of the IgG assays are very low (40.9% for the IFI with noncocultivated *B. henselae* as antigen) and that those of the IgM assays are higher (71.4% for the ELISA) for patients who fulfilled two or more criteria for CSD. The IgM ELISA showed the highest sensitivity: 71.4% in patients with two or more criteria for CSD and 80.6% for patients with a positive *Bartonella* PCR result. According to the authors, the results indicate that the specificities of both IFI and EIA IgG serologies and the sensitivity of the IFI IgM serology need to be improved; and more, major improvements may also be expected from the use of purified antigens in serologic assays.

6.3 PCR

The sensitivity of *Bartonella* spp. DNA amplification directly from patient samples (blood, CSF, synovial fluid, and cavity infusions) with active infection is low (BREITSCHWERDT, 2017). In addition, the use of conventional PCR for detection of *Bartonella* spp. in subclinical reservoirs also has a limited sensitivity. This occurs due to low loads of *Bartonella* in wild animals, resulting in false negative results. In addition, it is suggested to use more than one PCR technique, such as nested or real-time PCR, to improve diagnostic sensitivity (GUTIÉRREZ et al., 2017). In this context, molecular assays were rapidly adopted to improve sensitivity and facilitate identification. Many conserved and housekeeping loci have been developed as targets for the characterization and molecular detection of *Bartonella* organisms (Table 2). The targets used are: NADH dehydrogenase gamma subunit (*nuoG*) (ANDRÉ et al., 2015); type IV secretion system (*VirB4*) (KIMURA et al., 215); hypervariable region of the 16S-23S rRNA gene intergenic transcribed spacer (*ITS*) (PITASSI et al., 2015); the cell division protein gene (*ftsZ*) and riboflavin synthase gene (*ribC*) (PAZIEWSKA et al.,

2011); the pap31 bacteriophage associated gene (*Pap31*) (DINIZ et al., 2007); RNA polymerase beta-subunit-encoding gene (*rpoB*) (RENESTO et al., 2001); RNA componente of the 30S small subunit of a prokaryotic ribosome (*16S RNA*) (BERGMANS et al., 1995); cytrate synthase gene (*gltA*) (NORMAN et al., 1995).

In the study of André et al. (2015), they showed high sensivity and specificity using a new TaqMan assay in feline blood samples, detecting as few as 10 copies os plasmid/reaction and showed no croos-reaction with DNA of other pathogens analyzed. Kimura et al. (2015), diagnosed CSD by using real-time PCR with blood samples of a 6-year-old child, where antibody titers against *B. henselae* using IFA were 1:64 for IgG and < 1:10 for IgM. Regarding blood transmission, Pitassi et al. (2015), investigated *Bartonella* spp. prevalence in 500 blood donors from Campinas, Brazil. Blood samples were inoculated into a BAPGM and sub-inoculated onto blood agar. Liquid culture samples were tested using ITS PCR. *B. henselae* and *B. quintana* antibodies were assayed by indirect immunofluorescence. *B. henselae* was isolated from six donors (1.2%). Sixteen donors (3.2%) were *Bartonella*-PCR positive after culture in liquid or on solid media, with 15 donors infected with *B. henselae* and one donor infected with *Bartonella clarridgeiae*. Antibodies against *B. henselae* or *B. quintana* were found in 16% and 32% of 500 blood donors, respectively.

7. New diagnostic perspectives

The variability in the sensitivity of diagnostic methods to detect *Bartonella* species has a major influence on the results and interpretation of epidemiological studies, as seronegative bacteremia can result in underestimation of *Bartonella* seroprevalence in animals and humans; the ability of researchers to establish the cause of the disease; or the ability of physicians to reach a diagnosis to initiate

appropriate treatment (BREITSCHWERDT, 2017). In this context, studies have emerged from different parts of the world to improve the diagnosis of *Bartonella*. Research ranges from bacterial cultivation techniques to the production of recombinant proteins for use in serological diagnosis.

Several studies have emerged to identify antigenic proteins and/or propose a serological diagnosis of *Bartonella* spp. (Table 3) as GroEL, 17 kDa, Pap 31, SucB, P26 and BadA; in addition to vaccine research - still nonexistent to combat *Bartonella*. These studies are used as whole cell antigens, fractionated proteins and/or purified proteins. In this context, Ferrara et al. (2014) indicate the use of purified proteins as antigens to improve the sensitivity and specificity of serological tests.

GroEL and 17 kDa proteins were evaluated by Ferrara et al. (2014) to develop an ELISA-type serological test. In the study, the GroEL protein was identified through a proteomics approach. The results specificities found by the researchers were 89.1% and 93.5% for 17 kDa and GroEL, respectively. By combining the results from the two tests, the sensitivity of the test was 82.8% and the specificity 83.9%.

The use of recombinant proteins for the purpose of diagnosing *B. bacilliformis* infections has also been performed. In this case, Pap 31 protein was tested in a recombinant form on an ELISA and the results were compared with gold standard IFA. According to the authors, the test can differentiate naturally infected humans from non-infected humans (ANGKASEKWINAI et al., 2014).

The recombinant protein SucB (dihydrolipoamide succinyltransferase enzyme) was produced and tested against sera from rats immunized against *B. henselae* in a WB (KABEYA et al., 2003). In addition to this study, SucB was also tested against sera from patients with CSD previously confirmed by IFI. The agreement obtained between the tests was 55% and 88% with the use of positive and negative sera in IFA,

respectively. However, cross reactions with *Brucella melitensis*, *Mycoplasma pneumoniae*, *Francisella tularensis*, *Coxiella burnetii* and *Ricketsia typhi* (LITWIN et al., 2004) were observed.

Werner et al. (2008) tested the recombinant protein P26 in a capture ELISA against cat sera with a history of exposure to *Bartonella* spp. In addition to the indication of the recombinant protein in a serological test against *B. henselae* infections, the authors also indicate its use in cases of *Bartonella clarridgeiae* infection.

Lysed whole cells of *B. henselae* were used to obtain the BadA protein, and this was tested in a WB against serum from patients infected with *B. henselae* and these were recognized, suggesting the inclusion of this protein as a diagnostic input (WAGNER et al., 2008).

Jost et al. (2018) identified 16 fractions of *B. henselae* by ion change chromatography, where of these, the fraction called "24" was selected and tested in an ELISA against human sera. The reported sensitivity was 100% in cases of molecular proven infection, and 76% in cases of clinically suspected infections; the specificity for both cases was 93%. Most of the identified antigens have been tested in ELISA or WB assays, since serological assays using specific immunoreactive proteins instead of whole or lysed cells may have higher specificity due to the absence of cross-reactions, since they are highly purified antigens and are also very sensitive. In addition, these assays are advantageous in avoiding contact with infectious material (LITWIN et al., 2004; WERNER et al., 2008).

8. Conclusion

As for the correct understanding of any disease, highly sensitive and specific diagnostic tools are required. Faced with this, the identification of *B. henselae* as the

cause of disease still remains a difficult task, since the IFI (gold standard) suffers from cross reactions; cultivation for isolation requires prior enrichment (BAPMG is an alternative but must be followed by PCR); and the PCR technique has its sensitivity diminished when used in isolation. Given these limitations, the discovery of new antigens that can be used in serological tests is of great value; it will contribute to the understanding of the real importance of CSD. In this context, new antigens appear to be good targets, as GroEL, 17 kDa, Pap 31, SucB, P26 and BadA and should be better evaluated for use in diagnosis.

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Table 1: Prevalence of *Bartonella* spp. in Brazilian states.

State	Assay	Host	Number of Samples	Prevalence (%)	Reference
Maranhão	*PCR	Cats	200	4.5	(Braga et al., 2012)
Pernambuco	**IF	Dogs Cats	109 40	24.7 15	(Fontalvo et al., 2017)
Bahia	***ELISA	Mice	26	19	(Costa et al., 20140
Mato Grosso	PCR	Cats	178	2.2	(Miceli et al., 2013)
Mato Grosso do Sul	PCR	Cats	151	30.4	(André et al., 2015)
Minas Gerais	IF	Human	437	26.5	(Costa et al., 2005)
Rio de Janeiro	PCR	Human	1	100	(Favacho et al., 2014)
Rio de Janeiro	PCR	Cats	89	24.7	(Silva et al., 2017)
Rio de Janeiro	IF PCR	Cats	40	47.5 42.5	(Crissiuma et al., 2011;
Rio de Janeiro	IF	Cats	189	56.6	(Kitada et al., 2014)
Rio de Janeiro	PCR	Human	51	3.9	(Lamas et al., 2013)
São Paulo	IF PCR	Dogs	197 198	3.5 0.5	(Diniz et al., 2007)
São Paulo	PCR	Cats	46	4.3	(Bortoli et al., 2012)
São Paulo	Culture IF PCR	Human	500	3.2	(Pitassi et al., 2015; Vieira-Damiani et al., 2015)
São Paulo	Culture PCR	Human	1	100	(Drummond et al., 2019)
São Paulo	PCR	Cats	112	90.2	(Drummond et al., 2018)
Rio Grande do Sul	PCR	Cats	47	25.5	(Staggemeier et al., 2014)
Rio Grande do Sul	PCR	Cats	30	20	(Malheiros et al., 2016)

* Polymerase Chain Reaction; ** Immunofluorescence; *** Enzyme Linked Immuno Sorbent Assay.

Table 2: PCR-based methods used for detection of *Bartonella* spp.

Gene	PCR assay	Number and type of samples	Host	Reference
nuoG	qPCR**	151 Whole blood	Cats	(André etl al., 2015)
virB4	qPCR	1 Whole blood	Human	(Kimura et al., 2015)
ITS 314 – 357aa (16S-23S)	qPCR	500 Whole blood	Human	(Pitassi et al., 2015)
ribC *ftsZ* 16S RNA	PCR*	1457 Whole blood	Rodents	(Paziewska et al., 2011)
pap31	PCR	198 Whole blood	Dogs	(Diniz et al., 2007)
rpoB fragments	PCR followed RFLP***	94 Lymph node or pus aspirate	Human	(Renesto et al., 2001)
16S RNA	PCR	226 Pus aspirate, biopsies, pus aspirates of lymph node	Human	(Bergmans et al., 1995)
gltA	PCR followed RFLP	28 Bacterial strains	Cats	(Norman et al., 1995)

*Convencional Polymerase Chain Reaction; **Real-Time PCR; ***Restriction Fragment Length Polymorphism.

Table 3: Antigen candidates for serological diagnosis of *Bartonella* spp.

Antigen	Assay	Cross-reaction	Protein	Serum	Especifity/Sensitivity (%)	Reference
GroEL	ELISA	Not reported	Recombinant*	Human	82/42,9 IgG 97,7/45,3 IgM	Ferrara et al., 2014
17-kDa	ELISA	Not reported	Recombinant	Human	76/65,7 IgG 86,2/75 IgM	Ferrara et al., 2014
Pap31**	ELISA	No	Recombinant	Human	94/84,5 IgG 85,1/88,2 IgM	Angkasekwinai et al., 2014
SucB	WB	*Brucella melitensis* *Mycoplasma pneumoniae* *Francisella tularensi* *Coxiella burnetii* *Rickettsia typhi*	Recombinant	Human	88/55	Litwin et al., 2004
SucB	WB	Not tested	Recombinant	Mice	Not tested	Kabeya et al., 2003
P26***	WB	Not tested	Recombinant	Felin and Murine	Not tested	Werner et al., 2008
BadA	WB	No****	Whole cells lysates by SDS-PAGE*****	Human	74/74 IgG IgM not evaluated	Wagner et al., 2008
Fraction #24	ELISA	*B. quintana* *Brucella* spp. *Chlamydophila pneumoniae* *Coxiella burnetii* Epstein-Barr virus *Mycoplasma pneumoniae* *Treponema pallidum* *Rickettsia* spp. Rheumatoid fator Antinuclear antibodies	Line blotting and ion exchange chromatography	Human	93/100	Jost et al., 2018

*Recombinant: use of heterologous system for cloning and expression; ***B. bacilliformis;* ****B. henselae, B. koehlerae* and *B. clarridgeiae*. *B. henselae* in others studies.***** Tested only against *Yersinia enterocolitica* and *Y. pseudotuberculosis*; ****** Sodium dodecyl sulfate polyacrylamide gels.

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

Proteínas quiméricas recombinantes contendo epítopos antigênicos de *B. henselae* são promissoras no desenvolvimento de testes de diagnóstico.

3.2 Objetivo geral

Identificação "*in silico"* de epítopos antigênicos presentes em proteínas de *B. henselae* e utilização na construção de quimeras recombinantes para uso em diagnóstico.

3.3 Objetivos específicos

- Revisar a literatura científica na busca de proteínas de *B. henselae* relatadas como antigênicas;
- Realizar análises "*in silico*" para a identificação de epítopos antigênicos nestas proteínas;
- Desenhar genes quiméricos contendo os epítopos selecionados *"in silico"* para serem produzidos de forma sintética;
- Expressar e purificar as quimeras recombinantes utilizando sistema heterólogo baseado em *Escherichia coli*;
- Avaliar a antigenicidade das quimeras recombinantes produzidas utilizando soro de humano naturalmente infectado por *B. henselae.*

4. CAPÍTULOS

4.1 Manuscrito 2 – Immuno-bioinformatics-based approach for the construction of *Bartonella henselae* multi-epitope chimeras for use in diagnosis

Manuscrito submetido ao periódico *Current Microbiology*

Immuno-bioinformatics approach for the construction of *Bartonella henselae* multi-epitope chimeras for use in the diagnosis

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ABSTRACT

The diagnosis of *Bartonella henselae* remains a challenge. Bioinformatics tools have been used globally to optimize time in the search for new antigens. Through immuno-bioinformatics analysis, we identified the epitopes of *B. henselae* and built multi-epitope recombinant chimeras. The most important *Bartonella* antigens (GroEL, 17 kDa, P26, BadA, Pap31, OMP89, and OMP43) were chosen using two epitopes predictors. The three-dimensional study of proteins, structural quality analysis, physical-chemical analysis, antigenicity testing, the secondary structure of mRNA analysis was done. After, genes were chemically synthesized. The recombinant chimeras were inserted in an expression vector in prokaryotes. Analysis of the secondary structure of mRNA revealed that these were stable to translate the proteins. The chimeric proteins were expressed in a heterologous system and reacted with infected human

serum, with antigenicity and potential for use in diagnostic tests. We reported, for the first time, the production of recombinant chimeric proteins containing multi-epitopes of *B. henselae*.

Keywords: *in silico* analysis; recombinant protein; cat scratch disease; bartonellosis.

1. Introduction

Bartonella henselae is a Gram-negative, fastidious, facultative intracellular bacterium [1]. The bacterium is considered an important medical species because it infects cats (reservoirs) and humans (accidental hosts) [2]. The transmission of *B. henselae* to cats occurs directly through contact with fleas (*Ctenocephalides felis*) or through stool-infected wounds from these fleas, as well as through ticks (suggested as potential vectors) [2, 3]. Cats infected with *B. henselae* are often clinically asymptomatic, although they suffer from recurrent bacteremias for long periods. In humans, *B. henselae* is the etiological agent of cat scratch disease (CSD), a frequently self-limiting infection in immunocompetent individuals, but it can be fatal in immunocompromised individuals. Transmission to humans occurs through bites or scratches from flea-infested cats, which eliminate the bacteria in their stool, or directly through contaminated blood [3].

Bartonella species are found worldwide and are more pronounced in areas where conditions are more favorable for arthropod vectors, especially fleas [4]. In the United States, CSD is one of the most common zoonosis, resulting in more than 25,000 cases per year. In addition, seropositivity levels in healthy individuals are 19.6% in China, 16% in Sweden, and 8.7% in Spain [5]. Although there are some prevalence studies in Brazil, these may be few and may not portray reality in Brazil. However, the studies have indicated that the circulation of *Bartonella* species is common. Serologies show a prevalence of 15%-56.6% in cats [6–8] and 3.2%-23.5% in humans [9, 10]. Molecular methods have revealed the prevalence to be 2.2%-90.2% in cats [11–13] and 3.2%-100% in humans [9, 10, 14, 15]. With direct detection or antibody research, *B. henselae* has already been identified in cats in the states of Maranhão [16]; Pernambuco [8]; Bahia [17]; Mato Grosso [18]; Mato Grosso do Sul [19]; Rio de Janeiro [6, 7, 20]; São Paulo [13, 21], and the Rio Grande do Sul [11, 12]. Similarly, the microorganism has

been found in humans in the states of Rio de Janeiro [14, 15], São Paulo [10, 13], and Minas Gerais [22]. Interestingly, studies conducted in Brazil have demonstrated the presence of *Bartonella* spp. bacteremia in asymptomatic blood donors, reinforcing the need for evaluation of *Bartonella* blood transmission [9, 10].

The precise diagnosis of infection (reservoirs and hosts) can be challenging, especially in patients with chronic and long-term infection. Conventional techniques for detecting bacteria or antibodies against bacteria, such as enzyme-linked immunosorbent assay (ELISA), Western blotting (WB), indirect immunofluorescence (IFA), and polymerase chain reaction (PCR), have limitations [2]. IFI is the gold standard serological method for detecting antibodies against *B. henselae*, and it uses antigens from the entire bacterial cell co-cultivated in Vero cells, which have good sensitivity, but are expensive, laborious and can have cross-reactions [23] with *B. quintana*, *Coxiella burnetti*, *Chlamydophila pneumoniae, Ehrlichia chaffeensis*, *Mycoplasma pneumoniae*, *Escherichia coli*, *Rickettsia* spp., *Treponema pallidum, Bordetella pertussis*, *and Borrelia* spp. [24]. In the ELISA test, whole-cell antigens are used, but they have low sensitivity and specificity [23].

Rationally designed antigens can improve the sensitivity and specificity of diagnostic tests [23]. Outer membrane proteins (OMPs), which are an interface between the bacterium and host cells, may be targets in the development of diagnostic tests and vaccines. *B. henselae* OMP43 and OMP89 proteins play an important role in adherence and invasion of host cells [25]. Adhesin BadA, another OMP, may also be another important target, as this protein is recognized by antibodies from patients infected with *B. henselae* [26]. In addition, the use of recombinant proteins has been evaluated, and known antigenic proteins such as GroEL [27], 17kDa [23, 28, 29] , Pap31 [30], and P26 [31] have already been tested in serological assays. However, all evaluated proteins were used isolated, and in some of them, sensitivity was limited. This fact reinforces the need for the development of new alternatives, as chimeric proteins may be more immunogenic antigens than whole antigens.

Chimeric proteins are a new group of recombinant antigens that have recently been preferred over native recombinant antigens [32]. In addition, the use of synthetic peptides for

immunodiagnostic has been widely evaluated, leading to the development of several high sensitivity and specificity tests against pathogens in recent years. In the present postgenomic era, the availability of complete genome sequences and advanced analysis algorithms have contributed to the possibility of predicting antigens *in silico*, which could be useful for designing new immunodiagnostic tests [33].

2. Materials and Methods

2.1. Protein selection and *in silico* analysis

For choosing targets, we searched the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) to identify *B. henselae* OMPs. The search terms and the number of studies found were as follows: *Bartonella henselae proteins* (19); *Bartonella henselae outer membrane protein* (35); *Bartonella henselae antigens* (151); *Bartonella henselae recombinant proteins* (30); and *Bartonella henselae immunogenic proteins* (9). The summary of these studies was read, and all studies that identified *B. henselae* antigenic proteins were read in full to identify the targets. The criterion adopted for protein selection was the highest frequency of citation in the articles. Thus, the following proteins were selected: GroEL (Accession no. CUH91264), 17 kDa (Accession no. AAF00943), p26 (Accession no. ABB8349), BadA (Accession no. AAT69970), Pap31 (Accession no. AAC39274), OMP 89 (Accession no. CAF27432), and OMP 43 (Accession no. CAF27934).

The InterPro software (http://www.ebi.ac.uk/interpro/search/sequence/) was used for the functional annotation of proteins. *In silico* analysis was performed using predictors available online to identify the linear B cell epitopes in antigens of *B. henselae*. The selected regions contained 1 to 5 epitopes with 11 to 44 amino acids. The predictors used were as follows: Immune Epitope Database and Analysis Resource (www.iedb.org) and Sequential B-Cell Epitope Predictor 2.0 (http://www.cbs.dtu.dk/services/BepiPred/). The choice of two predictors served to increase the reliability of the selected epitopes. The selected epitopes were analyzed for the presence of orthologs in *B. henselae* using the Basic Logical Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Prediction of the tertiary structure

The sequences resulting from the translation of each chimera were also submitted to analysis on the *I-TASSER server* [34] for the prediction of its three-dimensional structure. The model generated with the largest C-Score was used for structural quality analysis using the QMEAN6 program [35]. Three-dimensional structures were visualized using the PyMol v1.8.4.0 tool [36].

2.3. Prediction of the secondary structure of mRNA

The RNAFold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) was employed to predict the secondary structure of RNA. This software predicts minimum free energy structures and base pair probabilities from single RNA sequences.

2.4. Physical and chemical characteristics

The ProtParam online server (http://us.Expasy.org/tools/protparam) was used to evaluate physicochemical parameters, including amino acid composition, theoretical isoelectric point, molecular weight (MW), *in vitro* and *in vivo* half-lives, aliphatic index, instability index, grand average of hydropathicity (GRAVY), and the total number of positive and negative residues.

2.5. Prediction of protein solubility

Protein solubility was predicted by using the SOLpro server at http://scratch.proteomics.ics.uci.edu/. SOLpro is used to predict the propensity of a protein to be soluble after overexpression in *E. coli* by using a support vector machine architecture based on multiple representations of the primary sequence [37].

2.6. Prediction of protective antigens

Antigenicity was predicted by using the VaxiJen server (https://omictools.com/vaxijen/tool). VaxiJen is used to predict protective antigens and subunit vaccines [38].

2.7. Production of recombinant chimeras

Sequences of the identified epitopes were used for three-chimera *in silico* designing. These sequences were later sent to GenOne (Rio de Janeiro, Brazil) for chemical synthesis, and the genes were cloned to the expression vector pAE and called as follows: pAE/*Chimera*1, pAE/*Chimera*2, and pAE/*Chimera*3. The vectors were transformed into *E. coli* BL21 Star (DE3) strains and cultivated in 500 mL of Luria-Bertani broth at 37°C at 200 rpm, until reaching DO_{600nm} = 0.5–0.7. Expression was then induced with 1 mM of isopropyl-β-1-D-tiogalactopyranosid. The cultures were kept under the same incubation conditions for another 3.5 h and then subjected to centrifugation (7000 x *g*, 4°C, 15 min). The cells were then suspended in a solubilization buffer (8 M urea, 200 mM NaH_2PO_4, 0.5 M NaCl, and 5 mM imidazole, pH 8.0) and incubated at room temperature at 60 rpm for 18 h. Purification was performed by chromatography by using HisTrap FF (GE Healthcare) columns loaded with nickel. The purified proteins were dialyzed against phosphate-buffered saline (PBS) 1X in 16 steps for five days at 4°C. The concentration of proteins was determined through the BCA Protein Assay kit (Pierce, USA), and the proteins were stored at −20°C. The purity and size of proteins were analyzed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE 15%) stained with Coomassie Blue. The expression of recombinant chimeras was confirmed by WB, by employing an anti-6× His-tag monoclonal antibody (Sigma-Aldrich, USA). Briefly, the chimeric proteins were separated by 15% SDS-PAGE and electro-transferred onto Hybond^{TM} ECLTM (Amersham Biosciences) nitrocellulose membranes. The membranes were blocked with PBS-FBS 1% (PBS with 1% [v/v] fetal bovine serum) and reacted with the anti-6×His-tag monoclonal antibody (Sigma-Aldrich, USA) at 1:100 dilution in PBS. Then, a goat anti-mouse Ig peroxidase conjugate (Sigma-Aldrich, USA) was added at 1:4000 dilution. Incubations were performed for 1 h at room temperature in agitation (50

rpm), and washes with PBS-T (PBS with 0.05% [v/v] Tween 20) were performed between all steps. Then, reactions were developed with a chromogen/substrate solution (6 mg diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris-HCl, pH 8.0, and 0.03% hydrogen peroxide) for the visualization of protein bands.

2.8. Evaluation of antigenicity of recombinant chimeras

A serum sample of a human naturally infected with *B. henselae* and previously tested by IFA for *B. henselae* was provided by the Division of Dermatology, Department of Medicine of the State University of Campinas, Campinas, São Paulo, Brazil. This serum sample was used for evaluating the antigenicity of recombinant chimeras produced. Briefly, the purified chimeras were submitted to 15% SDS-PAGE and electrotransferred onto a HybondTM ECLTM (Amersham Biosciences) nitrocellulose membrane. The membranes were blocked with PBS-FBS 1% at 4°C overnight and washed three times with PBS-T and incubated with human serum at 1:50 dilution (at room temperature for 1 h, under the agitation of 50 rpm). The membrane was then washed three times with PBS-T and incubated with the secondary human anti-IgG antibody conjugated to peroxidase at 1:1000 dilution in PBS-T. Reactions were developed with a chromogen/substrate solution (6 mg diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris-HCl, pH 8.0, and 0.03% hydrogen peroxide) for the visualization of protein bands.

3. Results

Among the several antigenic proteins mentioned in the literature, we selected seven for *in silico* analyses and determined their function through the InterPro software (Table 1). Subsequently, *in silico* analyses of the proteins were performed to seek epitopes involved in antibody production. Through alignment and search for similarity with the BLAST tool, we identified that these epitopes had 100% identity with those from *B. henselae*. Next, all the selected epitopes were randomly distributed and combined for chimera construction (Table 2) to form

three distinct recombinant chimeric proteins: chimera 1 (rC1), chimera 2 (rC2), and chimera 3 (rC3) (Figure 1). Between each epithet, linkers containing 3x glycine and 1x serine were added.

The RNAfold software was used for predicting the secondary structures of the mRNA of each chimera. The minimum free energy of the secondary mRNA structure was $\Delta G = -278.00$, -328.00, and -299.00 kcal/mol for rC1, rC2, and rC3, respectively. All structures lacked hairpins or pseudo-nodes on the side of 5′ end (Figure 2).

For evaluating physicochemical properties, we used the ProtParam online server to evaluate amino acid composition, theoretical isoelectric point, molecular weight, *in vivo* half-life, aliphatic index, instability index, extinction coefficient, grand average of hydropathicity, and the total number of positive and negative residues. Solubility was evaluated by using the Solpro server. The antigenic potential was evaluated by using the VaxyJen server; it is the first server for alignment-independent prediction of protective antigens of bacterial, viral, and tumor origin. All these parameters are presented in Table 3.

Table 4 presents the quality analysis of the model generated for the chimeras. The RMSD, TM-score values were calculated during modeling by the I-TASSER server. QMEAN6-score, Ramachandran plot, Z-score, and DFIRE-energy values were calculated in the QMEAN analysis program hosted by the SwissProt database. QMEAN analysis program also provides a visual quality analysis of the evaluated model. The analysis of local error by residue showed a general predominance of residues, indicating a low-quality prediction of the three-dimensional position (data not shown). The least reliable regions are the loopings and the 6× His tail, places that are difficult to predict because of the lack of a defined standard secondary structure.

The three recombinant chimeras (rC1, rC2, and rC3) were efficiently expressed and purified in *E. coli*. All proteins were insoluble expressed proteins and recovered with a denaturing agent. WB with anti-histidine antibodies confirmed that the chimeric proteins expressed were the recombinants. The antigenicity of the chimeric proteins was evaluated in a WB assay with the serum of a human naturally infected with *B. henselae*, demonstrating that the chimeras reacted with antibodies generated against native proteins (Figure 3). Compared with rC2 and rC3, rC1 reacted weakly.

4. Discussion

In this study, we reported, for the first time, the production of recombinant chimeric proteins containing multiepitopes of *B. henselae*. This was made possible by using bioinformatics tools, widely used worldwide, which reduce time and costs in laboratory analysis. These tools are advantageous because conventional methods require the cultivation of pathogens to extract their antigenic proteins. Although some bacteria grow fast (*B. henselae* has fastidious growth), the large-scale extraction and testing of bacterial proteins are expensive and laborious [39].

Several proteins have been reported in the literature as *B. henselae* antigens, demonstrating a great possibility of research that can be done for the development of new diagnostic and vaccine inputs. We chose seven proteins (GroEL, 17 kDa, P26, BadA, Pap31, OMP43, and OMP89), whose antigenicity was proven in at least one study. In addition, some of the proteins used have great importance during *B. henselae* infection and are reported as important antigens. The GroEL family of proteins is considered one of the main antigens of pathogenic bacteria [40]. The 17kDa antigen can be expressed at considerable levels during *B. henselae* infection [41]. The P26 protein is the largest immunodominant antigen expressed in experimentally infected cats [42], and OMP89 is one of the most immunogenic proteins *of B. henselae* [43].

Some studies have been conducted in different regions of the world in search of the best *B. henselae* antigen for use in diagnosis and vaccine. Studies with recombinant antigens have gained ground as *B. henselae* shows a cross-reaction with other microorganisms such as *B. quintana*, *Coxiella burnetti*, *Chlamydophila pneumoniae*, *Ehrlichia chaffeensis*, *Mycoplasma pneumoniae*, *E. coli*, *Rickettsia* spp., *Treponema pallidum*, *Bordetella pertussis*, and *Borrelia* spp.. This makes a species-specific diagnosis through serological tests difficult [24, 44]. Litwin et al. [45] produced the protein SucB (dihydrolipoamide succinyltransferase enzyme) in a recombinant form (rSucB). This recombinant form was tested against sera from patients with IFA-confirmed CSD, and the agreement obtained between the tests was 55% for IFA-positive sera and 88% for IFA-negative sera. However, cross-reactions with *Brucella melitensis*, *M. pneumoniae*, *Francisella tularensis*, *C. burnetii*, and *Rickettsia typhi* were observed. Wagner et al. [26] lysed whole *B. henselae* cells to obtain the BadA protein, and this was tested in WB

against the sera from patients infected with *B. henselae*. The protein was recognized, suggesting its inclusion as a diagnostic biotechnological input. Ferrara et al. [23] developed recombinant antigens from GroEL and 17kDa proteins in an ELISA assay and obtained good results, with a specificity of 93.5% and 89.1%, respectively. By combining the results, sensitivity was 82.8% and specificity 83.9%. Research for vaccine antigens is ongoing. Werner et al. [46] experimentally infected six SPF (free of specific pathogens) cats with *B. henselae,* and the antibodies produced recognized the recombinant P26 antigen within three weeks of infection. The results suggest that P26 can be developed for serological testing and should be considered as a potential vaccine antigen.

In this study, the WB performed with the sample of a human naturally infected with *B. henselae* showed a reaction with the three recombinant chimeras. However, compared with rC2 and rC3, rC1 reacted weakly. rC1 is composed of three proteins considered important antigens of *B. henselae* (as described above), but it is possible that the regions of the selected epitopes were not recognized by the antibodies present in the sample, and the portion of 17kDa was responsible for much of the response obtained. Similarly, the opposite is also possible, i.e., the epitopes of GroEL and/or P26 were recognized by the antibodies, but those of 17kDA were not identified. The latter is possible since GroEL and P26 make up rC2 and rC3, respectively, and these, in turn, showed a strong reaction in WB. However, the strong reactions shown by rC2 and rC3 could possibly be due to the recognition of components of other epitopes (BadA, Pap31, OMP43, and OMP89).

Evaluation of the quality of protein structures is an important part of experimental structure validation, playing a valuable role in predicting protein structure. Because predicted models may contain substantial errors, reliable estimates of absolute quality are crucial to assess the suitability of a model for specific biomedical applications [47]. In the quality analysis provided by the QMEAN6 program, which provides information on maintaining the native protein structure through positive scores, the scores obtained for chimeras 1, 2, and 3 were –13.1, –8.77, –17.31, respectively. These scores show that all chimeras obtained low scores (negative values), with rC2 being slightly better than the others. In the quality analysis of the models obtained for the three-

dimensional structures predicted by the I-TASSER server (data not shown), performed by the QMEAN6 program, it was possible to observe the local error per residue, configuring potentially unreliable regions, which represents a low-quality indicator prediction of the three-dimensional position of these residues in the predicted model for the protein. In addition, the graphs for the Z-score for each individual component considered in the QMEAN6-score calculation revealed the predominance of red color, indicating a bad score for all proteins. Only rC2 had a slightly better score than the others. The TM-score, which assesses the structural similarity of proteins, had values of 0.29 (rC1 and rC3) and 0.13 (rC2), meaning less structural similarity, instead of models with correct topology. In addition, the score values obtained by the RMSD for rC1 and rC3 were 16, whereas that for rC2 was 7.1, meaning that rC2 has a greater structural similarity in relation to the others.

RNA performs numerous cellular functions; thus, it becomes important to understand its structure to understand the mechanism of action. The secondary structure is the set of canonical base pairs, and the secondary structure can be accurately determined by comparative sequence analysis or can also be predicted [48]. The expression of high levels of recombinant proteins in *E. coli* is desired, and the stability of mRNA is crucial to this success. The secondary structure is a major factor in protein expression, and the results of mRNA prediction by the RNAfold server indicated that mRNA had sufficient stability for effective translation. Greater stability consequently leads to a higher expression rate. Although the expression levels seem to be the same as seen in Figure 3, in WB analysis, it is not possible to check the same efficiency, suggesting that rC1 has the least stable construction.

Analysis of the physicochemical parameters by the ProtParam server indicated that rC1 and rC2 were soluble. However, they proved to be insoluble *in vitro*, which perhaps can be attributed to the wrong pH used in protein purification. The isoelectric points of rC1 and rC2 were 6.57 and 5.12, indicating that the proteins are acidic, and that of rC3 was 9.14, indicating that the protein is basic. The instability indices of the three proteins were 32.3, 34.3, and 23.67, indicating that the proteins were stable. The aliphatic indices of the three proteins were 80.27, 67.77, and 71.39;

the greater the value, the higher is protein thermostability. The GRAVY values of the three proteins were −0.76, −0.57, and −0.71; a value greater than zero indicates a hydrophobic protein.

GroEL, 17kDa, P26, BadA, Pap31, OMP43, and OMP89 proteins are important antigens of *B. henselae* as described in the literature and should be tested for diagnostic and vaccine use. We reported for the first time the development of three chimeric proteins containing multi-epitopes of *B. henselae* obtained in a recombinant form. These recombinant chimeric antigens, built from *in silico* analysis, were recognized by antibodies generated during natural infection and can serve as biotechnological inputs for use in the diagnosis and control of CSD.

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Author's contributions

DDH and LSP conceived and designed research. JM, LSP and RW performed *in silico* analysis. JM, RW and SBF conducted experiments. DDH, JM and LSP contributed to the redaction of the manuscript. All authors read and approved the manuscript.

Conflict of interest

DDH, JM and SBF are inventors of patent protecting the recombinant chimeric protein (*Instituto Nacional de Propriedade Industrial* - BR1020190275740).

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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Table 1. Proteins selected and functional annotation extracted from the *InterPro* software.

Antigen	Molecular function/Biological process	Reference
GroEL	Protein refolding and folding/ATP-binding protein	[27,39,40,23]
17kDa	UNK	[41,28,29,23]
P26	UNK	[42,31,39]
BadA	Pathogenesis	[39,25]
Pap31	Transmembrane transport/Porin activity	[40,25]
OMP43	UNK	[25]
OMP89	Assembly membrane	[25]

GroEL: chaperone; P26: major immunodominant antigen; BadA: *Bartonella* adhesin A; Pap31: heme-binding protein; OMP: outer membrane protein; UNK: unknown.

Table 2. Selected epitopes of the GroEL, 17kDa, P26, BadA, Pap31, OMP89 e OMP43 proteins.

Antigen	Inicial position of the epitope	Final position of the epitope	Amino acid sequence
GroEL	322	346	kvniskenttiidgagqkseinarvn
	351	373	vqieettsdydreklqerlakla
	382	418	ggatevevkekkdrvddalnatraaveegivagggta
17kDa	1	115	tatltdeyykkalentqkldvaksqtaesiyesatqtankikdinnqlanlkadtktkpeqlqalqieltllqaqlqadtlkiqslamiqakdtktkeelreeqtqkkhedlqkq
	32	49	tqdktaqkaladnnksmn
	57	68	nngiqandlqts
	70	87	lsiyqsnpnkdhekknng
P26	103	109	Lsnagki
	118	123	Ftnantkpfyqe
	126	137	tgaknlsqnspgvnyskgshgsivlsgdddfcgady
BadA	34	142	vlgrggnstvrngipisveeeyerfvkqklmnnatspysqsseqqvwtgdgltskgsgymggkstdgdknilp
	5	19	phevaptvisapafs
	25	61	Iggqvgnfsskveitdpnkkdklfskddtpkpsgfmg
Pap31	64	71	yagsnmdl
	82	107	davwadredaktssaeaigqdeletf
	432	443	vternlggrgq
	449	457	glgagqeks
	464	473	fvdpyflgyr
OMP89	480	495	styradkaydvrqtg
	505	509	ndqls
	516	537	yiqeeydfgkkydlsketdire
	32	49	tqdktaqkaladnnksmn
	57	68	nngiqandlqts
	70	87	lsiyqsnpnkdhekknng
OMP43	103	109	lsnagki
	118	123	vnsvhg
	126	146	ftnantkpfyqearkkaiaea

Table 3. Evaluation of physicochemical parameters of chimeras.

ExPASy	**rC1**	**rC2**	**rC3**
Amino acids	337	328	346
Molecular Mass (kDa)	36900.11	34643.09	38220.29
Isoelectric Point (pI)	6.57	5.12	9.14
Negative residues (Asp + Glu)	45	46	34
Positive residues (Arg + Lyz)	43	33	40
Half Life (h) *E. coli*	>10	>10	>10
Index of instability	32.3 (stable)	34.30 (stable)	23.67 (stable)
Aliphatic index	80.27	67.77	71.39
Average hydropaticity (Gravy)	-0.760	-0.578	-0.716
Solubility	0.658302 (soluble)	0.553400 (soluble)	0.802264 (insoluble)
VaxiJen	-0.4924 (antigenic)	-0.4577 (antigenic)	-0.5928 (antigenic)

Table 4. Quality analysis of the three-dimensional structure model predicted, performed for the three chimeras.

Program	GroEL, 17kDa and P26	BadA, Pap31 and GroEL	OMP89, OMP43 and P26
QMEAN6 score	-13,41	-8.77	-17.31
C-score	-3.97	-0.31	-4.01
TM-score	0.29±0.09	0.67±0.13	0.29±0.09
RMSD	16.4±3.0	7.1+-4.1	16.6+-2.9

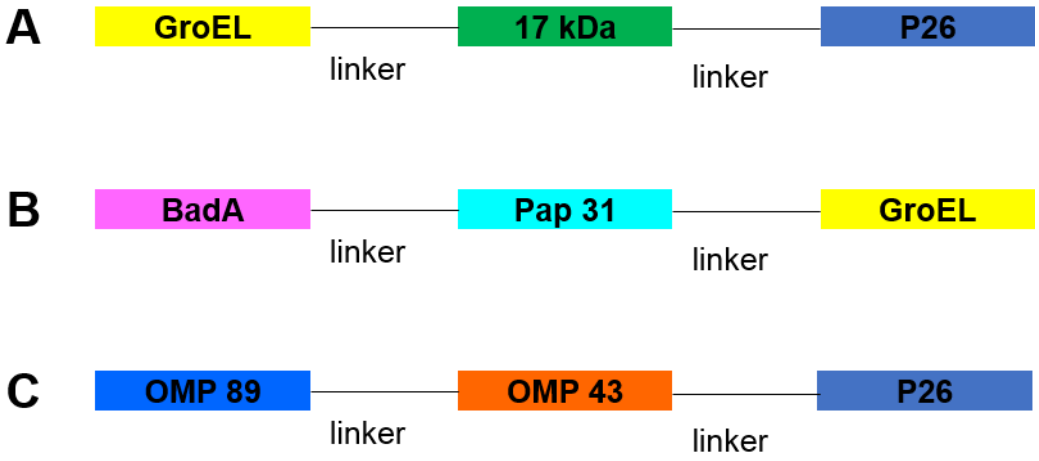

Figure 1. Final sequence of the rC1 (A), rC2 (B) and rC3 (C). Linker: GGGS.

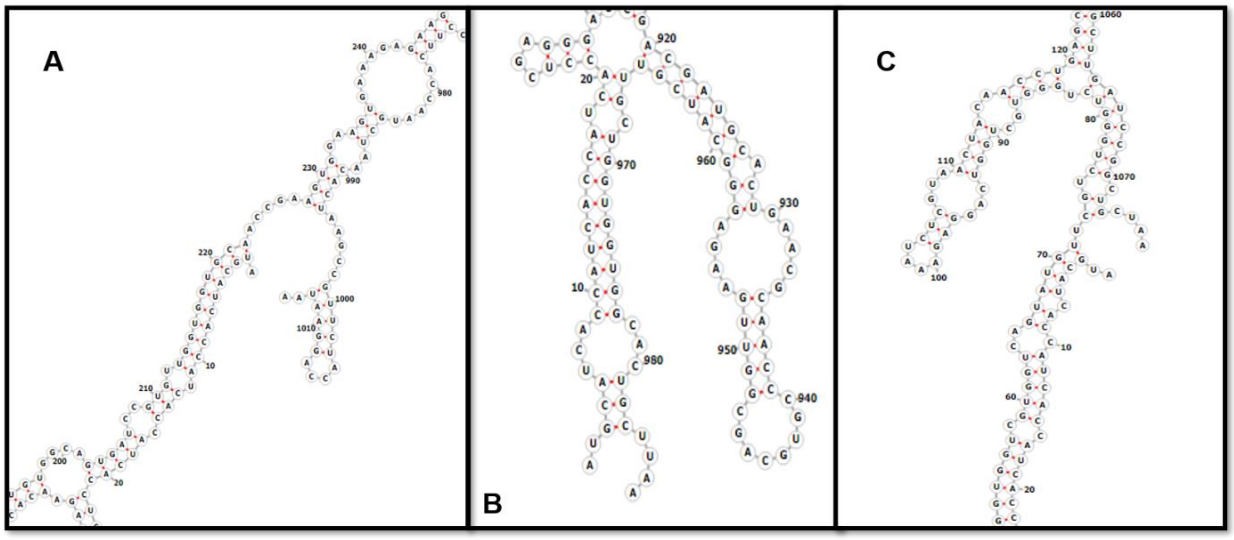

Figure 2. The ideal secondary structure of the mRNA of rC's 1, 2 and 3 in the point support notation with a minimum free energy of -278.60 (A), -328.20 (B) and -278.60 (C) kcal/mol are given above, respectively. The predicted structure has no hairpin and pseudo node at the 5' mRNA site.

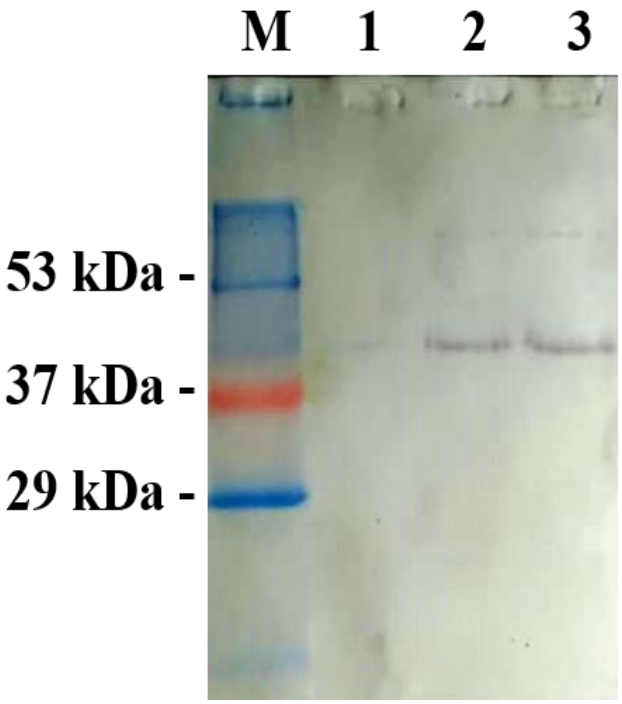

Figure 3. WB of serum from a patient naturally infected with *B. henselae* to evaluate the antigenicity of the three chimeric proteins. M: Pre-stained protein molecular weight marker (Ludwig); 1: rC1 (37 kDa); 2: rC2 (35 kDa) and 3: rC3 (38 kDa).

4.2 Patente – Proteínas quiméricas recombinantes contendo antígenos de *Bartonella henselae*

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PROTEÍNAS QUIMÉRICAS RECOMBINANTES CONTENDO ANTÍGENOS DE *Bartonella henselae*

RESUMO

A presente invenção enquadra-se nos setores técnicos A61K 39/02 (antígenos bacterianos), C12N 15/09 (tecnologia do DNA recombinante), C12N 15/10 (processos para purificação, isolamento ou purificação de DNA ou RNA), C12N 15/31(genes que codificam proteínas microbianas), C12N 15/66 (métodos gerais para inserção de um gene em um vetor para formar um vetor recombinante usando clivagem ou ligação), C12N 15/70(vetores ou sistemas de expressão especialmente adaptados à *E. coli*), C12N 15/74 (vetores ou sistemas de expressão especialmente adaptados a procariotos além de *E. coli*) e G01N 33/53 (imunoensaio). O invento refere-se à construção de três proteínas quiméricas sintéticas, a partir de epítopos imunogênicos de diferentes antígenos de *Bartonella henselae*. Estas quimeras são produzidas em sistemas de expressão heteróloga e servem para o desenvolvimento de testes de diagnóstico e vacinas recombinantes no controle da bartonelose humana e animal.

PROTEÍNAS QUIMÉRICAS RECOMBINANTES CONTENDO ANTÍGENOS DE *Bartonella henselae*

RELATÓRIO DESCRITIVO

CAMPO DA INVENÇÃO

[001] A presente invenção, enquadrada internacionalmente nas classificações de patentes A61K 39/02 (antígenos bacterianos), C12N 15/09 (tecnologia do DNA recombinante), C12N 15/10 (processos para purificação, isolamento ou purificação de DNA ou RNA), C12N 15/31(genes que codificam proteínas microbianas), C12N 15/66 (métodos gerais para inserção de um gene em um vetor para formar um vetor recombinante usando clivagem ou ligação), C12N 15/70(vetores ou sistemas de expressão especialmente adaptados à *E. coli*), C12N 15/74 (vetores ou sistemas de expressão especialmente adaptados a procariotos além de *E. coli*) e G01N 33/53 (Imunoensaio) refere-se à construção de genes quiméricos sintéticos para produção de proteínas quiméricas, contendo epítopos imunogênicos de diferentes proteínas de *Bartonella henselae.* Estas proteínas quiméricas serão empregadas como antígenos em testes de diagnóstico da bartonelose causada por *B. henselae*, mais conhecida como Doença da Arranhadura do Gato.

DESCRIÇÃO DO ESTADO DA TÉCNICA

[002] *Bartonella henselae* é uma bactéria que pertence à família *Rhizobiales*, é uma α-Proteobacteria gram-negativa de crescimento fastidioso, intracelular facultativa e pode infectar os eritrócitos e as células endoteliais de seus hospedeiros **(GIL, H., ESCUDERO, R., PONS, I., RODRIGUEZ-VARGAS, M., GARCÍA, E., RODRÍGUEZ-MORENO, I., et al. Distribution of *Bartonella henselae***

variants in patients, reservoir hosts and vectors in Spain. Plos One., v. 8, p. 1-10, 2013). Os gatos (*Felis catus*) são considerados reservatórios primários para *B. henselae*, entretanto, cães (*Canis familiaris*) também têm sido apontados como reservatórios, enquanto os seres humanos são considerados hospedeiros acidentais **(BREITSCHWERDT, E. Bartonellosis, One Health and all creatures great and small. Vet. Dermatol., v. 28, p. 96-e21, 2017).** Dentro do gênero *Bartonella*, existem em torno de 22 espécies relacionadas com doenças em mamíferos **(PENNISI, M.G., MARSILIO, F., HARTMANN, K.,et al. *Bartonella* species infection in cats – ABCD guidessline on prevention and managment. J. Fel. Med. Surg., v.15, p. 563-569, 2013)**, e *B. henselae* é considerada uma das espécies de maior importância médica pois infecta tanto gatos como seres humanos **(BREITSCHWERDT, E. Bartonellosis, One Health and all creatures great and small. Vet. Dermatol., v. 28, p. 96-e21, 2017).**

[003] A transmissão de *B. henselae* para humanos ocorre através de mordidas ou arranhaduras de gatos infectados ou contaminados com pulgas contendo a bactéria em suas fezes, ou ainda, diretamente através de sangue contaminado. Em humanos, *B. henselae* é o agente etiológico da Doença da Arranhadura do Gato (*Cat Scratch Disease* – CSD), que é uma infecção frequentemente auto-limitante em indivíduos imunocompetentes **(REGIER, Y, O'ROURKE, F., KEMPF, V.A.J. Bartonella spp. – a chance to establish One Health concepts in veterinary and human medicine. Parasites Vectors., v. 9, p. 1-12, 2016)**, e tem como principal sintoma a linfadenopatia regional **(GIL, H., ESCUDERO, R., PONS, I., RODRIGUEZ-VARGAS, M., GARCÍA, E., RODRÍGUEZ-MORENO, I., et al. Distribution of *Bartonella henselae* variants in patients, reservoir hosts and vectors in Spain. Plos One., v. 8, p. 1-10, 2013).** Outros sintomas podem envolver febre, dor de cabeça, lesões na pele e mucosas próximas ao sítio de inoculação e

esplenomegalia. Endocardites, envolvimento oculoglandular (Síndrome de Parinaud), encefalopatias, neuroretinites e osteomielites são descritas como complicações da infecção. Em indivíduos imunocomprometidos, infecções crônicas podem ocorrer, levando a doenças angioproliferativas como a angiomatose bacilar e peliose hepática, que podem ser fatais se não tratadas **(REGIER, Y, O'ROURKE, F., KEMPF, V.A.J. Bartonella spp. – a chance to establish One Health concepts in veterinary and human medicine. Parasites Vectors., v. 9, p. 1-12, 2016).** [72]

[004] As espécies de *Bartonella* possuem distribuição mundial, encontrando-se de forma mais pronunciada em áreas onde as condições são mais favoráveis para vetores artrópodes, principalmente pulgas **(PENNISI, M.G., MARSILIO, F., HARTMANN, K.,et al. *Bartonella* species infection in cats – ABCD guidessline on prevention and managment. J. Fel. Med. Surg., v.15, p. 563-569, 2013).** Em áreas endêmicas para presença de pulgas, os níveis de soroprevalência de *Bartonella* spp. em gatos podem ser maiores que 90%, e os níveis de bacteremia maiores que 50%. Inicialmente, *B. henselae* foi isolada de um indivíduo HIV positivo, e, posteriormente, de gatos ao redor do mundo. Nos dias atuais, a bacteremia por *B. henselae* tem sido reportada nas mais diferentes espécies, como vacas, cavalos, mamíferos marinhos, pequenos mamíferos terrestres e tartarugas marinhas, fazendo a epidemiologia desta espécie ser mais complexa do que incialmente descrita **(BREITSCHWERDT, E. Bartonellosis, One Health and all creatures great and small. Vet. Dermatol., v. 28, p. 96-e21, 2017).** No Brasil, estudos epidemiológicos têm demonstrado a presença das espécies de *Bartonella*. A detecção deste patógeno vai desde gatos até seres humanos, nos mais diversos Estados brasileiros. Interessantemente, recentes estudos no Brasil têm demonstrado a presença de bacteremia por *Bartonella* spp. em doadores de sangue assintomáticos, reforçando a necessidade de avaliação da transmissão de *Bartonella* pelo sangue **(PITASSI,**

L.H.U., DINIZ, P.P.V.P., SCORPIO, D.G., DRUMMOND, M.R., LANIA, B.G., BARJAS-CASTRO, M.L., et al. *Bartonella* spp. Bacteremia in blood donors from Campinas, Brazil. Plos Negl. Trop. Dis., v. 9, p. 1-2, 2015; VIEIRA-DAMIANI, G., DINIZ, P. P.V., PITASSI, L.H.U., SOWY, S., SCORPIO, D.G., LANIA, B.G., DRUMMOND, M.R., SOARES, T.C.B., BARJAS-CASTRO, M.L., BREITSCHWERDT, E.B., NICHOLSON, W.L., VELHO, P.E.N.F. *Bartonella clarridgeiae* bacteremia detected in a asymptomatic blood donor. J. Clin. Microb., v. 53, p. 352-356, 2015).

[005] Um diagnóstico microbiológico preciso de bartonelose pode ser um grande desafio, principalmente em pacientes com infecção crônica e de longa duração. Técnicas convencionais de isolamento, ELISA (*Enzyme Linked Immuno Sorbent Assay*), *Western Blotting* ou Imunofluorescência para detecção de anticorpos e Reação em Cadeia da Polimerase (PCR) para amplificação de DNA bacteriano têm demonstrado limitações diagnósticas **(BREITSCHWERDT, E. Bartonellosis, One Health and all creatures great and small. Vet. Dermatol., v. 28, p. 96-e21, 2017).** Ademais, a detecção sorológica de *B. henselae* também é difícil e é prejudicada pela sensibilidade e especificidade baixas dos ensaios utilizados na rotina laboratorial **(OTSUYAMA, K., TSUNEOKA, H., KONDOU, K., et al. Development of a Highly specific IgM enzyme-linked immunosorbent assay for *Bartonella henselae* using n-lauroyl-sarcosine-insoluble proteins for serogianosis of cat scratch diaseae. J. Clin. Microbiol., v. 54, p. 1958-1964, 2016).** Nos testes sorológicos, a detecção é feita por imunofluorescência indireta usando antígenos de células inteiras co-cultivadas em células Vero, que tem boa sensibilidade, porém são caros e podem ter reações cruzadas. No método de ELISA, antígenos de células inteiras têm sido empregados, porém, além da baixa sensibilidade, apresentam pouca especificidade, neste sentido, o uso de proteínas purificadas poderia melhorar a sensibilidade e especificidade destes testes **(FERRARA, F., DI**

NIRO, R., D'ANGELO, S., BUSETTI, M., MARCARI, R., NOT, T., SBLATTERO, D. Development of an enzyme-linked immunosorbent assay for *Bartonella henselae* infection detection. Letters Applied Microbiol., v. 59, p. 253-262, 2014). Alguns estudos com proteínas recombinantes têm sido publicados. Nestes, proteínas reconhecidamente antigênicas, como GroEL e 17-kDa **(FERRARA, F., DI NIRO, R., D'ANGELO, S., BUSETTI, M., MARCARI, R., NOT, T., SBLATTERO, D. Development of an enzyme-linked immunosorbent assay for *Bartonella henselae* infection detection. Letters Applied Microbiol., v. 59, p. 253-262, 2014)**, Pap31 **(ANGKASEKWINAI, N., ATKINS, E.H., ROMERO, S., GRIECO, J., CHUNG CHAO, C., CHING, W.M. An evaluation study of enzyme-linked immunosorbent assay (ELISA) using recombinant proten Pap31 for detection of antibody against *Bartonella bacilliformis* infection among the peruvian population. Am. J. Trop. Med. Hyg., v. 90, p. 690-696, 2014)** e P26 **(WERNER, J.A., FENG, S., CHOMEL, B., HODZIC, E., KASTEN, R.W., BARTHOLD, S. P-26 based serodiagnosis for *Bartonella* spp. infection in cats. Comparative Med., v. 58, p. 375-380, 2008)** têm sido produzidas de forma recombinante, e têm sido testadas em ensaios sorológicos, principalmente ELISA. Entretanto, em todos os ensaios as proteínas foram utilizadas de forma isolada, e, de forma geral, apresentaram baixa sensibilidade, o que indica que o uso de proteínas purificadas como antígenos pode melhorar a sensibilidade e especificidade dos testes de diagnóstico **(FERRARA, F., DI NIRO, R., D'ANGELO, S., BUSETTI, M., MARCARI, R., NOT, T., SBLATTERO, D. Development of an enzyme-linked immunosorbent assay for *Bartonella henselae* infection detection. Letters Applied Microbiol., v. 59, p. 253-262, 2014)**, bem como, poderiam ser usadas em vacinas.

[006] Desta forma, o desenvolvimento de insumos para o diagnóstico sorológico e produção de vacinas para a infecção por *B. henselae* é de considerável interesse, tanto na medicina humana quanto na veterinária, contribuindo para o correto tratamento

das enfermidades causadas por *B. henselae*, bem como, para o real conhecimento da epidemiologia da infecção. Neste contexto, se reforça a necessidade do desenvolvimento de quimeras como alternativas para o diagnóstico e tratamento da infecção, pois estas proteínas quiméricas são constituídas por diferentes antígenos que compõe as proteínas bacterianas, e isto poderia tornar os ensaios mais sensíveis e específicos, e seria possível obter uma resposta eficaz na administração de vacinas, visto que anticorpos poderão ser produzidos frente aos diferentes antígenos quiméricos. Com base nisto, nossa proposta é utilizar análise *in silico* para identificar proteínas antigênicas capazes de compor um teste sorológico eficaz e vacinas, baseado na utilização destas proteínas recombinantes na forma de quimeras.

SUMÁRIO DA INVENÇÃO

[007] Neste âmbito, os eventos descritos a seguir, apresentam a invenção relativa a proteínas quiméricas baseadas em diferentes combinações de epítopos de diferentes antígenos de *B. henselae*, visando a utilização destas proteínas como insumos no desenvolvimento de testes de diagnóstico.

[008] O primeiro objetivo da presente invenção trata da construção de sequências de DNA para síntese química de genes quiméricos que codificam epítopos de diferentes antígenos de *B. henselae.* Outro objetivo refere-se à produção das proteínas quiméricas recombinantes em sistema de expressão heteróloga para utilização das mesmas como insumo no diagnóstico da bartonelose.

DESCRIÇÃO DETALHADA DO INVENTO

[009] Por conveniência o significado de alguns termos empregados nas especificações estão descritos abaixo.

[010] Q1: refere-se à sequência de nucleotídeos (SEQ ID N° 1) ou à sequência de aminoácidos (SEQ ID N°4) da quimera 1, composta por porções das proteínas GroEL, 17kDa e P26.
[011] Q2: refere-se à sequência de nucleotídeos (SEQ ID N° 2) ou à sequência de aminoácidos (SEQ ID N°5) da quimera 2, composta por porções das proteínas BadA, Pap31 e GroEL.
[012] Q3: refere-se à sequência de nucleotídeos (SEQ ID N° 3) ou à sequência de aminoácidos (SEQ ID N°6) da quimera 3, composta por porções das proteínas OMP89, OMP43 e P26.

[013] Sob um aspecto, a invenção trata da construção de sequências de DNA sintéticas que codificam proteínas quiméricas compostas epítopos de diferentes antígenos de *B. henselae*. As etapas de construção e clonagem quimeras estão detalhadas abaixo.

[014] **Passo 1:** Na presente invenção a identificação dos antígenos foi baseada na análise *in silico* de antígenos de *B. henselae* previamente descritos na literatura. Os preditores *online* de antígenos de células B utilizados foram o *BepiPred-2.0: Sequential B-Cell Epitope Predictor* e *Immune Epitope Database and Analysis Resource*.

[015] **Passo 2:** As sequências nucleotídicas que codificam epítopos envolvidos com a estimulação de linfócitos B foram utilizados para construir os genes quiméricos Q1 (SEQ ID N° 1), Q2 (SEQ ID N° 2) e Q3 (SEQ ID N° 3) e estes sintetizados pela empresa GO Genone®. Todos os genes foram clonados em plasmídeos pAE que possuem as seguintes características: origem de replicação em *Escherichia coli,* sítio de múltipla clonagem, gene de resistência ao antibiótico ampicilina e promotor do fago T7, que é reconhecido pela RNA polimerase de *E. coli*. Além disso, este vetor permite a expressão das proteínas recombinantes

fusionadas a uma cauda de seis resíduos de histidinas em sua porção amino-terminal. Entre as sequências de cada epítopo foram utilizados "linkers" contendo 3x o aminoácido glicina e 1x o aminoácido serina. O códon de iniciação foi o aminoácido metionina. Todas as sequências foram digeridas com as enzimas de restrição *BamH*I (região N-terminal) e *Kpn*I (região C-terminal).

[016] Sob um segundo aspecto, a invenção trata da obtenção e caracterização das proteínas quiméricas recombinantes, compostas por epítopos de diferentes antígenos de *B. henselae*. As etapas de produção e caracterização das proteínas quiméricas estão detalhadas abaixo.

[017] Para expressão das proteínas quiméricas Q1 (SEQ ID N° 4), Q2 (SEQ ID N° 5) e Q3 (SEQ ID N° 3) fusionadas a uma cauda de seis histidinas, os vetores recombinantes pAE/Q*1,* pAE/Q2 e pAE/Q3 foram utilizados para transformar *E. coli* BL21(DE3) Star através de choque térmico (15 min a 4°C, 1 min a 2°C e 2 min a 4°C). Um clone recombinante para cada construção foi utilizado para inocular 10 mL de LB contendo 100 µg.mL^{-1} de ampicilina e cultivado sob agitação de 180 rpm, por 12 – 20 h a 37 °C. Esta cultura foi utilizada para inocular 500 mL de LB com 100 µg.mL^{-1} de ampicilina, que foi incubado a 37 °C e agitação de 180 rpm até a fase exponencial de crescimento (DO_{600} entre 0,6 e 0,8), quando então a expressão das quimeras foi induzida com 1 mM de IPTG (isopropiltio-β-D-galactosideo), durante 3 h. Após este período, a cultura foi fracionada em tubos de 250 mL, centrifugada a 6.000 × *g* por 15 min a 4° C. O pellet foi suspendido em um tampão de solubilização (8 M de uréia, 0,1 M de Tris-base, 0,3 M de NaCl e 5 mM de imidazole, pH 8,0) e incubado em agitador orbital a 60 rpm por 18 horas, em temperatura ambiente. As células foram então sonicadas (3 ciclos de 30 s, 20 kHz), centrifugadas (10.000 × *g* por 30 min a 4 °C) e o

sobrenadante coletado e filtrado em membrana de 0,8 µM (Millipore). As proteínas quiméricas foram purificadas através de cromatografia de afinidade utilizando colunas Ni^{2+} Sepharose HisTrap e dialisadas de forma lenta, utilizando-se membrana de celulose para diálise (poros de 14 kDa) (Sigma). A diálise foi realizada a 4 °C contra tampão contendo 100 mM de Tris-base e 300 mM de Nacl em concentrações decrescentes de ureia. Por fim, a proteína foi dialisada contra tampão fosfato-salino (PBS) (pH 7,2) *overnight* a 4 °C e armazenada a – 20 °C. A concentração das proteínas purificadas foi determinada usando BCA Protein Assay Kit (Pierce, USA), com uma curva padrão de albumina sérica bovina (BSA).

[018] **Passo 3:** As proteínas recombinantes foram submetidas a Western Blot **(SAMBROOK, J.; RUSSEL, D.W. Molecular cloning: a laboratory manual. 2001. Cold Spring Harbor Laboratory Press, 3a Ed.)**, com anticorpo monoclonal anti-histidina (Sigma-Aldrich, USA) para verificação de sua expressão. As quimeras foram submetidas a um SDS-PAGE 10% e eletrotransferidas para membranas de nitroceluse Hybond™ ECL™ (Amersham Biosciences). As membranas foram bloqueadas com PBST acrescido de 5% de leite em pó desnatado, a 4 °C, 12 – 20 h e, após este período, lavadas 3 vezes com PBST. Posteriormente, as membranas foram incubadas durante 1 h à temperatura ambiente com MAb anti-histidina conjugado com peroxidase na diluição de 1:10000. Após 3 lavagens com PBST, as reações foram reveladas com diaminobenzidina (DAB) e H_2O_2. Os resultados podem ser observados na Figura 1.

[019] **Passo 4:** As proteínas recombinantes foram submetidas a Western Blot **(SAMBROOK, J.; RUSSEL, D.W. Molecular cloning: a laboratory manual. 2001. Cold Spring Harbor Laboratory Press, 3a Ed.)**, com soros de indivíduos naturalmente infectados com *B. henselae*, para verificação de sua antigenicidade. O protocolo

acima descrito (**Passo 3**) foi também utilizado nesta etapa. Entretanto, o anticorpo secundário utilizado foi o MAb anti-IgG humano conjugado com peroxidase na diluição de 1:10000. Os resultados podem ser observados na Figura 2.

[020] **Passo 5:** Para o uso destas proteínas quiméricas em diagnóstico sorológico, é necessário que soros controle padronizados sejam utilizados para reação com as quimeras recombinantes para o estabelecimento de ponto de corte de cada proteina avaliada, assim como especificidade e sensibilidade, o que resultará na acurácia do teste desenvolvido para cada quimera.

[021] **Passo 6:** Sob outro aspecto, a invenção trata da utilização das proteínas quiméricas como vacinas recombinantes (subunidades, vetorizadas e genéticas) contra bartonelose. As etapas de avaliação experimental desta aplicação seriam a imunização de ratos com as vacinas quiméricas recombinantes, avaliação da resposta immune humoral e celular e ainda capacidade imunoprotetora das vacinas, através de testes de desafio e esterilidade.

Breve Descrição das figuras

A Figura 1 ilustra a expressão das proteínas quiméricas recombinantes Q1, Q2 e Q3, produzidas em *E. coli*, por *Western blotting* utilizando anticorpo monoclonal anti-histidina. M: Marcador de peso molecular de Proteínas Pré Corado (Ludwig); 1: Proteína rQ1(37 kDa); 2: Proteína rQ2 (35 kDa); 3: Proteína rQ3 (37 kDa).

A Figura 2 ilustra a caracterização antigênica das quimeras recombinantes através de Western blot com soro humano

naturalmente infectado por *B. henselae*. M: Marcador de peso molecular de Proteínas Pré Corado (Ludwig); 1: Proteína rQ1(37 kDa); 2: Proteína rQ2 (35 kDa); 3: Proteína rQ3 (37 kDa).

FIGURAS

Figura 1

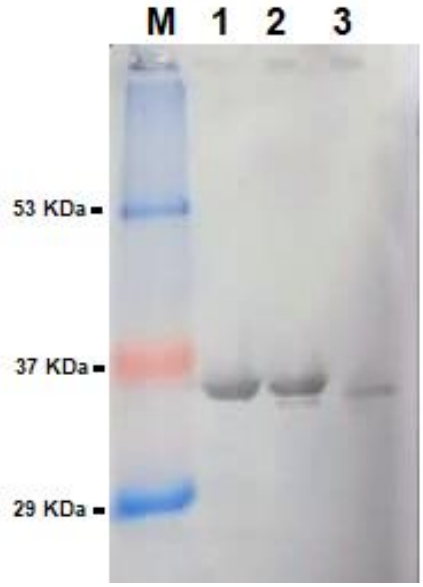
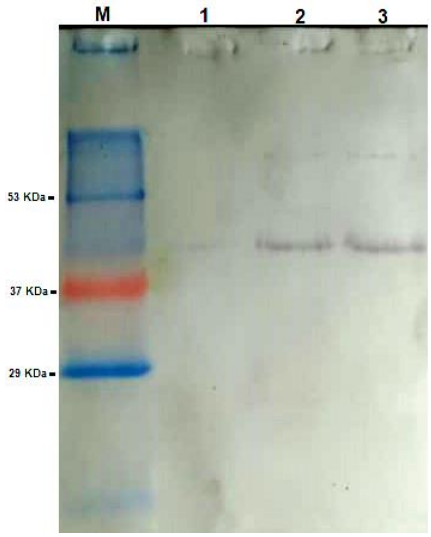


Figura 2



REIVINDICAÇÕES

1) Proteínas quiméricas recombinantes contendo antígenos de *Bartonella henselae* **caracterizadas por** sequências nucleotídicas (SEQ ID N° 1, SEQ ID N° 2 e SEQ ID N° 3) presentes nas proteínas GroEL, 17kDa, P26, BadA, Pap31, OMP43 e OMP89 de *Bartonella henselae*;

2) Vetores recombinantes contendo sequências nucleotídicas conforme reivindicação 1, **caracterizados por** serem utilizados para clonagem ou expressão das proteínas quiméricas (SEQ ID N° 4, SEQ ID N° 5 e SEQ ID N° 6) em procariotos, eucariotos ou mamíferos, mas não por estes limitada;

3) Processo de produção das proteínas quiméricas recombinantes conforme reivindicação 1 **caracterizado por** utilizar vetor recombinante de expressão em *Escherichia coli*, seguido ou não da purificação das proteínas;

4) Proteínas quiméricas recombinantes obtidas conforme reinvindicações 1, 2 e 3 **caracterizadas por** serem utilizadas de forma individual ou combinada em testes de diagnóstico sorológico da bartonelose;

5) Proteínas quiméricas recombinantes obtidas conforme reinvindicações 1, 2 e 3 **caracterizadas por** compor formulações vacinais individuais ou combinadas, para induzir resposta imune específica contra *Bartonella henselae*.

5 CONCLUSÕES

- Na revisão da literatura científica foram identificadas sete proteínas antigênicas de *B. henselae* (GroEL, 17kDa, P26, BadA, Pap31, OMP89 e OMP43), sendo as mais citadas em artigos científicos;

- Através de análises "*in silico*" utilizando ferramentas de imuno-bioinformática foi possível a identificação e seleção de regiões contendo de 1 a 5 epítopos antigênicos com 11 a 44 aminoácidos nas proteínas GroEL, 17kDa, BadA, Pap31, P26, OMP43 e OMP89 de *B. henselae*;

- Todos os epítopos selecionados foram randomicamente distribuídos e combinados para a construção das quimeras, a fim de formar três distintas proteínas quiméricas recombinantes: Quimera 1 (rC1), Quimera 2 (rC2), e Quimera 3 (rC3);

- As análises de bioinformática da estrutura secundária do mRNA revelaram que estes são estáveis e funcionais na tradução das proteínas quiméricas;

- As análises de qualidade estrutural das quimeras revelaram uma baixa qualidade na predição dos epítopos;

- Foi possível expressar e purificar as quimeras recombinantes utilizando sistema heterólogo baseado em *Escherichia coli*;

- As quimeras recombinantes mostraram-se antigênicas sendo reconhecidas por anticorpos presentes no soro de humano naturalmente infectado por *B. henselae;*

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7 ANEXOS

ANEXO A – Pedido Nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2019 027574 0

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Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): PROTEÍNAS QUIMÉRICAS RECOMBINANTES CONTENDO ANTÍGENOS DE Bartonella henselae

Resumo: A presente invenção enquadra-se nos setores técnicos A61K 39/02 (antígenos bacterianos), C12N 15/09 (tecnologia do DNA recombinante), C12N 15/10 (processos para purificação, isolamento ou purificação de DNA ou RNA), C12N 15/31(genes que codificam proteínas microbianas), C12N 15/66 (métodos gerais para inserção de um gene em um vetor para formar um vetor recombinante usando clivagem ou ligação), C12N 15/70(vetores ou sistemas de expressão especialmente adaptados à E. coli), C12N 15/74 (vetores ou sistemas de expressão especialmente adaptados a procariotos além de E. coli) e G01N 33/53 (imunoensaio). O invento refere-se à construção de três proteínas quiméricas sintéticas, a partir de epítopos imunogênicos de diferentes antígenos de Bartonella henselae. Estas quimeras são produzidas em sistemas de expressão heteróloga e servem para o desenvolvimento de testes de diagnóstico e vacinas recombinantes no controle da bartonelose humana e animal.

Figura a publicar: 1

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Documentos anexados

Tipo Anexo	Nome
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Sequências Biológicas

☑ Declaro que a informação contida na 'Listagem de Sequências' apresentada em formato eletrônico está limitada ao conteúdo da matéria revelada pelas sequências de aminoácidos e/ou de nucleotídeos divulgadas no pedido de patente, conforme depositado

Tipos de Sequências Biológicas	Nome
Listagem de Sequências Biológicas em formato TXT	Sequencia biológica.txt

Acesso ao Patrimônio Genético

☑ Declaração Negativa de Acesso - Declaro que o objeto do presente pedido de patente de invenção não foi obtido em decorrência de acesso à amostra de componente do Patrimônio Genético Brasileiro, o acesso foi realizado antes de 30 de junho de 2000, ou não se aplica.

Declaração de veracidade

☑ Declaro, sob as penas da lei, que todas as informações acima prestadas são completas e verdadeiras.