DESIGN AND EVALUATION OF A MULTIEPITOPIC PROTEIN AS A CANDIDATE FOR A CARRION DISEASE VACCINE

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ABSTRACT

Objectives. To design and assess a multiepitopic protein as a candidate for a vaccine against Carrion disease. ***Materials and Methods.*** Using bioinformatics tools, epitopes of external membrane proteins were selected and a multiepitopic protein was designed. The multiepitopic protein gene was subcloned into the expression plasmid pET28b and transformed into E. coli BL21 pLys. The multiepitopic protein was expressed using isopropyl-β-D-1-thiogalactopyranoside and purified using resin. This purified protein was used to immunize BALB/c mice obtaining polyclonal antibodies. In vitro invasion assays were conducted using a strain of *Bartonella bacilliformis* (*B. bacilliformis*) in human red blood cells. ***Results.*** The multiepitopic protein M1 presents preserved epitopes between isolates of *B. bacilliformis* with are non-toxic, and not homologous to human and surface proteins. Immunized mice presented IgG antibody levels capable of reducing in vitro the rate of invasion of *B. bacilliformis* into human red blood cells. ***Conclusions.*** Multiepitopic protein M1 may serve as a candidate for a Carrion disease vaccine; however, more studies are needed to characterize the use of this antigen as a vaccine.

Keywords: Bartonella infections; Bartonella bacilliformis; Computational biology; Epitopes; DNA, recombinant; Immunogenicity, vaccine (source: MeSH NLM).

INTRODUCTION

Carrion disease (CD), caused by infection with *Bartonella bacilliformis* (*B. bacilliformis*), is a serious public health problem in Peru. The cases of this disease show cyclical trend increases according to reports from the Directorate General of Epidemiology (DGE). Outbreaks also occurred in different provinces of the departments of Áncash, Cajamarca, La Libertad, Amazonas, Cuzco, and Madre de Dios [1-5].

This disease is biphasic, the first phase is characterized by hemolytic anemia and fever that can cause death; and the second phase is more benign and is characterized by self-limited bleeding warty rashes. Control of this disease is based on vector control, early diagnosis and timely medication[2-5]; however, cases are still reported today.

Therefore, the development of a vaccine, which requires years of systematic antigen evaluation, is indispensable. An alternative to shorten and improve the antigen selection process in an efficient way is to use the available genomic information, together with immunoinformatic tools [6,7]. On the other hand, there is little information about possible vaccines for Carrion's disease [8].

The outer membrane protein (OMP) proteins of many pathogens are considered good candidates for vaccines. Immunization with these proteins could be efficient for the production of antibodies that would block infection in experimental models. OMP16, OMP19 and OMP31 proteins of *Brucella ovis* are immunodominant antigens, antibodies against these proteins confer an important protection against brucellosis in the murine model [9,10]. For *Chlamydia trachomatis* it has been reported that the purified MOMP protein can generate protection in murine model and protects against vaginal infection [11,12].

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It has also been shown that purified PMOs of *Neisseria lactamica* protect against infection of *Neisseria* spp serogroup B and C isolated from meningitis [13]. Other studies on OMP-based vaccines for *Neisseria meningitidis* group B have shown to be effective in inducing protective antibody-based response in healthy adults in Cuba, Brazil, Chile and Europe, with an efficacy between 50% to 80% [14-17]. *B. bacilliformis* has been reported to have at least 14 external membrane proteins, which have a molecular weight between 11 KDa and 75 KDa. Of these external membrane proteins, those with 31 KDa, 42 KDa and 45 KDa are the most antigenic [18].

In the present study, a multiepitope protein was designed and evaluated as a candidate for vaccination against Carrion disease.

KEY MESSAGES

Research motivation. The absence of a vaccine to prevent Carrion's disease in endemic areas of Peru generated the need to develop a vaccine proposal against this public health problem. Since there is no animal model for this disease, we approach an in vitro infection model.

Main Findings. The multiepitopic protein M1 is immunogenic in the murine model. In addition, antibodies generated against this protein inhibit the invasion of *Bartonella bacilliformis* to human red blood cells in *in vitro* assays.

Implications. This study contributes to the development of experimental vaccine proposals against Carrion's disease.

MATERIALS AND METHODS

This study is of quantitative experimental analytic nature.

EPITOPES SELECTION AND MULTIEPITOPES PROTEIN DESIGN

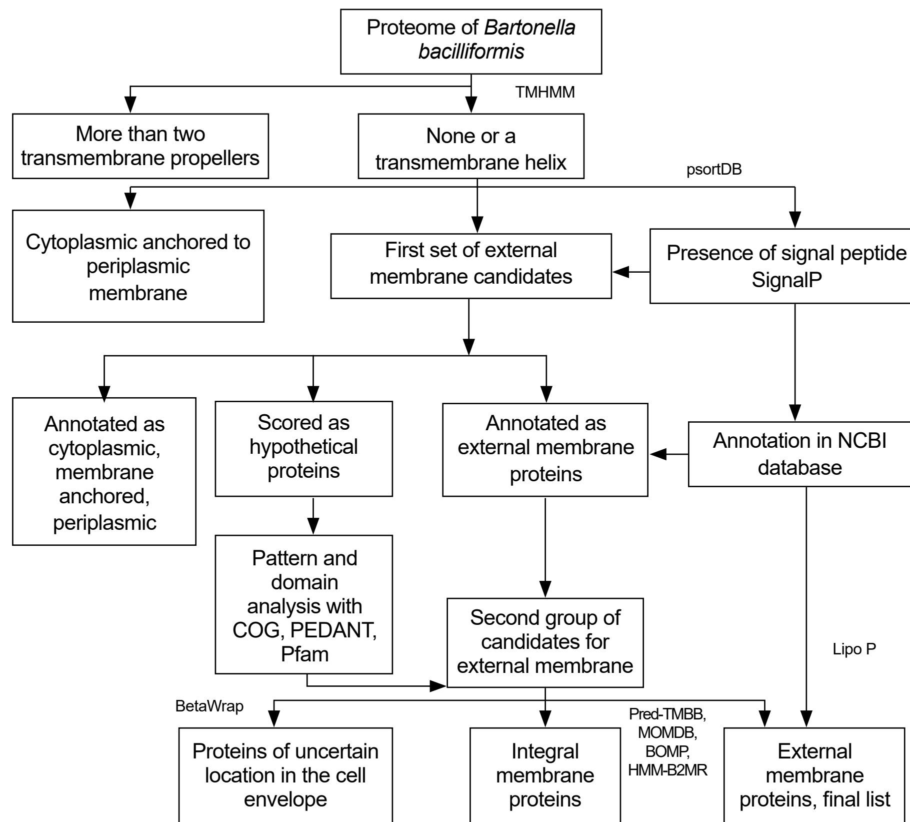
All proteins deduced from the genome of B. bacilliformis (strain KC583, GenBank access number CP000524) were used to select external membrane proteins; these proteins were considered as candidate antigens for the elaboration of a vaccine.

The proteins to be analyzed were selected according to the algorithm indicated in Figure 1. First, proteins with one or no transmembrane helix were selected. To investigate whether they contained a transmembrane alpha-helix region, TMHMM 2.0 server (http://www.cbs.dtu.dk/ services/TMHMM/) was used. The selected proteins were then evaluated for possible localization as external membrane proteins, using the cPsortdb database (http://db.psort.org), selecting whether they were membrane proteins or proteins whose localization could not be determined. Of those proteins whose cellular location could not be predicted, it was evaluated if they presented signal peptide using the SignalP 3.0 server program (http://www.cbs.dtu.dk/ services/SignalP/), selecting those that presented signal peptide. From this first set of proteins, those annotated as external membrane proteins or hypothetical proteins were selected.

From the group of hypothetical proteins, those that presented domains and patterns compatible with other external membrane proteins were selected, using the PEDANT database (http://pedant. gsf.de), Pfam (http://pfam.sanger.ac.uk/) and COG (http:// www. ncbi.nlm.nih.gov/COG) databases. A lipoprotein prediction was performed using the LipoP 1.0 server program (http://www.cbs.dtu.dk/services/ LipoP/). The BetaWrap program (http://groups.csail.mit.edu/ cb/betawrap/) was used to assess the likelihood of proteins forming beta helixes. Betabarrel Outer Membrane protein Predictor (BOMP) (http://www.bioinfo.no/ tools/ bomp), Prediction of TransMembrane Beta-Barrel Proteins server (PREDTMBB) (http://bioinformatics.biol. uoa.gr/PRED-TMBB/), Markov Chain Model for Beta Barrels prediction program (MCMBB) (http://athina.biol) were used to identify possible "beta-barrel" structures. uoa.gr/ bioinformatics/mcmbb/) and the B2TMR-HMM predictor (http://gpcr.biocomp.unibo.it/ predictors/).

Once the external membrane proteins had been selected, it was evaluated whether these proteins had epitopes binding to the major histocompatibility complex (MHC) I and II using default parameters in the netpanMHC (http://www.cbs.dtu.dk/ services/NetMHCpan/), and netpanMHCII (http://www. cbs.dtu.dk/ services/NetMHCIIpan/) programs. In addition, BIMAS (HLA Peptide Binding Predictions), SYPEITH (Epitope prediction), LBtope (Linear B-Cell epitope Prediction server - Imtech), BepiPred (BepiPred 1.0 Server), BCPREDs (B-cell epitope prediction server) and EIBD (Linear B cell epitope prediction tool) were used. An epitopes table was made according to a ranking of association with MHC I and II.

In order to determine the conservation of epitopes, the sequence of epitopes of all genomes reported by BLASTp (www.ncbi. nlm.nih.gov/BLAST) was compared. The toxicity of the epitopes was evaluated using the ToxinPred server (http://crdd.osdd. net/ raghava/toxinpred/). In addition, epitopes that are not homologous to human



NCBI: National Center for Biotechnology Information

Figure 1. Algorithm for the selection of external membrane proteins of *Bartonella bacilliformis*

protein epitopes were selected using BLASTp (http://www.ncbi.nlm.nih.gov/BLAST).

Epitopes were evaluated by measuring their accessibility index with the Emmi Surface Accessibility Prediction server (http://tools.immuneepitope.org/bcell). On the other hand, the proteins were modeled using the RaptorX server (http://raptorx.uchicago.edu) and their structures visualized with PyMol version 2.3 (https://pymol.org). The structure model obtained was evaluated according to the Ramachandra index (https://swift.cmbi.umcn.nl/ servers/html/ramchk.html).

During the design of the multiepitope protein, it was verified that they do not generate new epitopes that could bind to MHC using netpanMHC and netpanMHCII. Theoretical coverage was calculated using the Population Coverage program on the Epitope Prediction and Analysis Tools server (http://tools.iedb.org/population/). Six histidine residues were added to the amino acid sequence of the multiepitope protein at the terminal carboxyl end to allow purification by nickel-charged affinity resins. Then, from the amino acidic sequence of the multiepitope protein, inverse translation and optimization of codons for their adequate expression in Escherichia coli were carried out. Finally, the synthetic gene obtained was synthesized commercially (Integrated DNA Technologies Inc., USA).

CLONING, EXPRESSION, AND PURIFICATION OF MULTIEPITOPE PROTEIN IN Escherichia coli

The gene of the multiepitope protein synthesized in the plasmid pUC (Integrated DNA Technologies Inc., USA) was released by enzymatic digestion with the enzymes Xho I and Nco I, the released fragment was purified and linked to the expression plasmid pET28 (Invitrogen Inc, USA) previously digested with the same restriction enzymes.

To join the liberated fragment and the plasmid, the enzyme ligase T4 (Promega Corp., USA) was used, then *E. coli* BL21 pLys (Invitrogen Inc, USA) was transformed into competents prepared by the calcium chloride method[19]. The multiepitope protein was expressed by induction with 0.5 mM IPTG (isopropyl- β-D-1-thiogalactopyranoside)

(Sigma-Aldrich subsidiary Merck, USA), and then this protein was purified by chromatography using the nickel-charged nickel-charged resin Ni-NTA agarose (QIAGEN N.V., Germany) following the manufacturers' recommendations (Annex 1).

IIMMUNIZATION OF BALB/C MICE WITH MULTIEPITOPE PROTEIN

In order to obtain polyclonal antibodies, immunization assays were carried out using female BALB/c mice five to eight weeks of age. Fifteen mice were inoculated subcutaneously, the first dose consisted of 5 µg of multiepitope protein supplemented with complete Freund adjuvant (Sigma-Aldrich subsidiary Merck, USA) at a final concentration of 50%. Three reinforcements were then made with 5 µg of multiepitope protein supplemented with Freund's incomplete adjuvant (Sigma-Aldrich subsidiary Merck, USA). These reinforcements were administered on days 7, 14, and 21 after the initial inoculation.

A second group of 15 mice was immunized with the same scheme but using alumina as an adjuvant (Sigma-Aldrich subsidiary Merck, USA). For the control group, the same immunization scheme was followed with ten mice, replacing the multiepitope protein with sterile PBS (Phosphate-Buffered Saline) and using the complete Freund adjuvant for the first dose and incomplete for the reinforcements. 30 days after the first dose, the mice were slaughtered and whole blood was collected. After 30 minutes of coagulation, the sera were separated by centrifugation at 1700 rpm for 15 minutes. The sera were stored at -20°C until use.

ELISA

The ELISA (Enzyme-Linked ImmunoSorbent Assay) assay was performed according to the procedure reported previously with some modifications(20). The ELISA plates were impregnated with 100 ng of purified recombinant M1 antigen.

INVASION TEST

Human erythrocytes were obtained from volunteer donors (who were not born or traveled to endemic Carrion disease areas), these erythrocytes were washed twice with RPMI 1640 (Roswell Park Memorial Institute, Sigma-Aldrich) and quantified in Neubauer chamber. B. bacilliformis was grown in biphasic medium, harvested and resuspended in RPMI medium. The bacteria were quantified by spectrophotometry at 600 nm.

Approximately 106 bacteria (in a volume of 50 µL) were mixed with 50 µL of a pool of immunized mouse sera or with 50 µL of RPMI medium as a control and incubated for 30 minutes at 30ºC. After this incubation, the mixture was added to 100 µL of washed erythrocytes (105 erythrocytes), centrifuged at 500 g for five minutes and the vials were incubated for three hours at 30ºC. After this incubation, the free bacteria were separated from the erythrocytes using density gradient, 400 µL of ficoll-hypaque (Sigma-Aldrich) were added to a 1.5 mL sterile vial and on top of the ficoll-hypaque the sample of infected erythrocytes or controls was carefully added. These samples were centrifuged at 500 g for 30 minutes. The supernatant was removed, and erythrocytes were washed twice with RPMI. DNA was purified from the entire sample of infected red cells using the PureLink Genomic DNA (Invitrogen) purification kit following the manufacturer's recommendations with some modifications. The cells were lysed by incubating the lysis/proteinase K sample/buffer mixture at 65°C for one hour and the genomic DNA was eluted into 200 µL of elution solution.

To calculate the number of equivalent genomes present in the sample, a quantitative real-time polymer chain reaction (PCR) was performed, using for each reaction: 12.5 µL of Sybr Green Master Mix (Roche), 1 µL of 5 µM of forward primer (5'-ATGTSAATGGRAATTTAGGG-3'), 1 µL of 5 µM of reverse primer (5'-ATGTSAATGRAATTTAGG-3'), and 5 µL of purified DNA from each experiment. The following amplification program was applied: 95ºC for 10 minutes followed by 45 cycles of 95ºC for 20 s, 42ºC for 20 s, 50ºC for 20 s and 72ºC for 30 s. A standard curve was also made with purified genomic DNA from the ATCC strain (American Type Culture Collection) KC583 *B. bacilliformis*. Invasion trials were conducted in duplicate and repeated twice. On the other hand, the primers used were designed for the present study, these primers are sensitive and specific for the detection of *B. bacilliformis* by real-time PCR (unpublished data).

STATISTICAL ANALYSIS

Statistical analyses were performed using R Studio version 1.2.1335. The Mann-White U nonparametric test was used to assess the statistical significance of antibody titer means and invasion assays for independent random samples. P values less than 0.05 were considered statistically significant.

ETHICAL ISSUES

This project was approved by the Institutional Ethics Committee on Human Beings and by the Ethics Committee for Experimental Animals of the National Health Institute of Peru.

RESULTS

EPITOPES SELECTION AND MULTIEPITOPE PROTEIN DESIGN

The reported proteins of the complete genome of B. bacilliformis were 1,285 annotated proteins, these were downloaded from the Genbank international database (this annotation presents a provisional status). Of these proteins, 142 had a helix-type transmembrane domain (11.1%) and 979 proteins had none (76.3%) when evaluated with TMHMM server. Of these selected proteins, 24 were external membrane proteins (1.9%) and 365 proteins of unknown location (28.4%) when evaluated by pSORT. Then, the presence of signal peptide was evaluated in the list of proteins whose location could not be determined using the SignalP program, selecting 103 proteins. Thus, the first list of 127 antigen candidates was formed.

This first list was analyzed its annotation in the GENBANK and the databases PEDANT database, Pfam and COG were consulted, so ten cytoplasmic proteins and seven periplasmic localization proteins were depurated. Two proteins scored as external membrane proteins from the group of proteins of unknown location and which did not have a predictable signal peptide type domain were included. Also, this list of proteins was purified with BOMP, PREDTMBB, MCMBB and B2TMR-HMM programs. Thus, a final list of 112 proteins was obtained. This list includes three autotransport proteins, 25 external membrane proteins (five of which are annotated as lipoproteins), ten membrane-associated proteins, and 74 hypothetical proteins of unknown function.

The list of 112 external membrane proteins was used to select epitopes that are recognized by the major histocompatibility complex (MHC) class I and II corresponding to the Peruvian alleles. Eight programs were used to minimize false positives (netpanMHC, netpanMHCII, BIMAS, SYPEITH, LBtope, BepiPred, BCPREDS and EIBD), each with its own parameters for prediction. A list of epitopes was obtained which were prioritized. In addition, all epitopes had an accessibility index greater than 1.00 using Emmi Surface Accessibility Prediction. It was verified that all epitopes were on the surface of the structure of their RaptorX-modeled proteins. Also, the selected epitopes were non-toxic. The similarity of the selected epitopes with the sequence of the human genome was verified in order to avoid the sequence homology with the human protein avoiding the formation of autoantibodies using Standard Protein BLAST.

Conservation of epitopes was verified by comparing selected proteins with GenBank base strains Hosp800-02, Car600-02, VERO075, PERU38, VERO097, CUSCO5, Cond044, PERU-18, Str. Heidi Mejia and SanPedro600. The epitopes are listed in Table 1. The chosen combination of epitopes did not form new epitopes that bind to MHC,

Table 1. Epitopes selected to conform the multiepitope protein M1, these are recognized by alleles of the major complex of histocompatibility I and II present in the Peruvian population.

GenBank	Name	Conservation %	MHC II		MHC I	
			Position	Epitope	Position	Epitope
WP_005766369	ATP synthase subunit b1	80 -100 *	114	AIQEISSSAVNLAIS	--	No interacción
WP_005766764.1	Protein contained in the domain DUF1009	100	160	EKNSILLAAKAAKLL	165	LAAKAAKLL
WP_005766132.1	Peptidase of family M23	93.3 - 100 *	119	NFEWIRMALAEERLH	124	RMALAEERL
WP_005766132.1	Peptidase of family M23	86.6 - 100 *	211	NNEYFPILPFIDPLQ	--	No interacción
WP_011807308	Peptidase of family M23	93.3 - 100 *	254	QSQFSSNLSMQKSLQ	--	No interacción
WP_005766132.1	Peptidase of family M23	86.6 - 100 *	168	TQTKMTLRSRPLNTY	169	QTKMTLRSR
WP_005766203	Protein homóloga a Methyl-transferase	100	222	RILRADTAAVAALAI	223	ILRADTAAV
WP_005766517	Protein from the recipient family dependent on TonB	100	415	SRGFGLAFENEIDFY	418	FGLAFENEI
WP_005767319	LemA Family Protein	100	173	YNTALKTMPAMLWAK	176	ALKTMPAML
WP_005766357	Hypothetical external membrane protein	100	286	TPEKLIKAYLNAKKMK	285	TPEKLIKAY
WP_005766862	Hypothetical external membrane protein	100	204	DSANFARDGLIELLT	209	ARDGLIELL
WP_005767808	Hypothetical external membrane protein	93.3 - 100 *	88	LIHDAIFQNFWGSYS	89	IHDAIFQNF

MHC: major histocompatibility complex, No interaction: epitope does not interact with MHC
A: alanine, R: arginine, N: asparagine, D: aspartic acid, C: cysteine, Q: glutamine, E: glutamic acid, G: glycine, H: histidine, I: isoleucine, L: leucine, K: lysine, M: methionine, F: phenylalanine, P: proline, S: serine, T: threonine, W: tryptophan, Y: tyrosine, V: valine.
* The epitope shows amino acidic changes with respect to the Ver097 strain (very divergent strain considered as a possible subspecies).

the amino acid sequence of the multi-epitope protein M1 is presented in Figure 2.

The multiepitope protein M1 does not form complex secondary structures according to its theoretical model obtained with RaptorX (Figure 3). The multiepitope protein structure model presents a Ramachandra index of 98.4% (90.3% of residues in favorable position and 8.1% in permitted regions). Likewise, the theoretical coverage of a vaccine based on M1 multiepitope protein calculated by the Population Coverage program is 99.9% for the Peruvian population.

LEVELS OF ANTIBODIES OBTAINED IN MICE

Mice immunized with M1 protein supplemented with Freund adjuvant had higher IgG antibody levels (mean 2.107; standard deviation 0.141) than those immunized with M1 protein supplemented with alumina (mean 1.190; standard deviation 0.507). These two groups had higher IgG antibody levels than the control group (mean 0.506; standard deviation 0.190) (Figure 4). Statistical differences were evident between immunized group averages and control (p <0.005).

INHIBITION OF THE INVASION OF B. bacilliformis A HUMAN ERITROCYTES

The erythrocyte invasion control incubated with B. bacilliformis showed similar amounts of bacteria per assay (mean 2.25 x 105; standard deviation 0.126) than the group treated with control mouse sera immunized with PBS/Freund adjuvant (mean 2.253; standard deviation 0.206). While invasion assays treated with sera from the Freund M1/adjuvant immunized mouse group (mean 1.453; standard deviation 0.170) and the M1/adjuvant alumina group (mean 1.093; standard deviation 0.167) had lower bacterial values per assay, indicating that antibodies against the multiepitope protein contained in the sera inhibit invasion of *B. bacilliformis* to human erythrocytes in in vitro assays (Figure 5). In addition, greater inhibition is seen when used as an adjuvant to alumina. Statistical difference was evidenced between the averages of the groups treated with anti-M1 antibodies and the control (p <0.005).

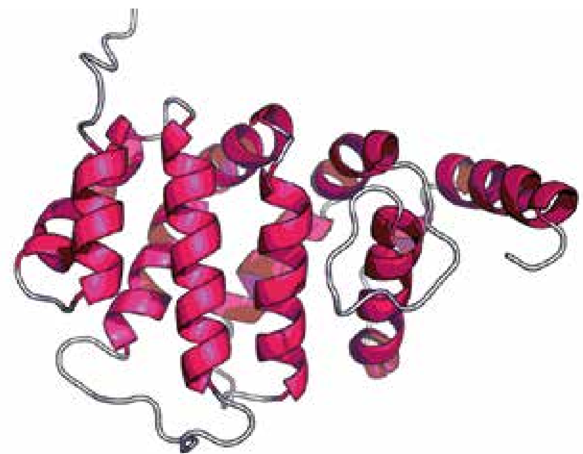


Figure 3. Three-dimensional model of the multiepitope protein M1 obtained with the X-raptor server, visualized in the PyMol program.

1 10 20 30 40 50
MAIQEISSSAVNLAISEKNSILLAAKAAKLLNFEWIRMALAEERLHNNEY
epitope 1 epitope 2 epitope 3

60 70 80 90 100
FPILPFIDPLQTPEKLIKAYLNAKKMKQSQFSSNLSMQKSLQRILRADTA
epitope 4 epitope 5 epitope 6 epitope 7

110 120 130 140 150
AVAALAISRGFGLAFENEIDFYTQTKMTLRSRPLNTY YNTALKTMPAMLW
epitope 8 epitope 9 epitope 10

160 170 180 188
AKDSANFARDGLIELLTLIHDAIFQNFWGSYSHHHHHH
epitope 11 epitope 12

Alanine (A); arginine (R); asparagine (N); aspartic acid (D); cysteine (C); glutamine (Q); glutamic acid (E), glycine (G); histidine (H); isoleucine (I); leucine (L); lysine (K); methionine (M); phenylalanine (F); proline (P); serine (S); threonine (T); tryptophan (W); tyrosine (Y); valine (V).

Figure 2. Amino-acid sequence of the multiepitope protein M1, made up of epitopes selected from Table 1, the number indicates the position of the amino acids in the protein sequence. At the end of the terminal carboxyl there are six histidine residues to facilitate purification.

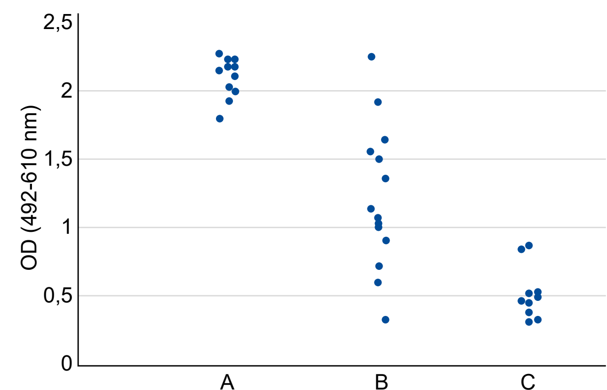
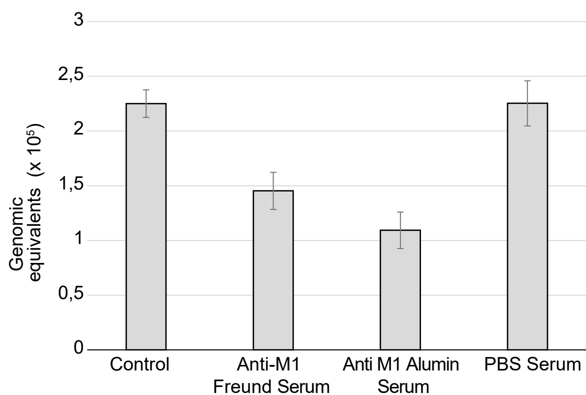


Figure 4. Mean serum anti-M1 IgG antibody levels of immunized BALB/c mice: A) immunized with M1 protein and Freund adjuvant, B) immunized with M1 protein and alumina adjuvant, and C) immunized with Phosphate-Buffered Saline (PBS) and Freund adjuvant as control.



PBS: Phosphate-Buffered Saline
M1: multiepitopic protein M1

Figure 5. Inhibition of *Bartonella bacilliformis* invasion to human erythrocytes in in vitro assay. Control: red blood cells infected with *Bartonella bacilliformis*. Freund anti-M1 serum: the bacterium was previously incubated with a *pool* of polyclonal anti-M1 serums from mice immunized with M1 protein and Freund adjuvant. Anti-M1 Alumin Serum: polyclonal anti-M1 sera of mice immunized with M1 and adjuvant Alumin. PBS serum: polyclonal sera from mice immunized with Freund's PBS plus adjuvant. The bars represent the average of genomic equivalents quantified after the invasion assay while the whiskers represent a range of twice the standard deviation.

DISCUSSION

The multiepitope protein M1 is immunogenic in the murine model. In addition, anti-M1 antibodies inhibit the invasion of *B. bacilliformis* to human red blood cells in *in vitro* assays.

Our study points to the development of a vaccine against Carrion's disease that would allow the reduction of multiepitope would allow the use of the best epitopes of the pathogen proteome dramatically increasing the quality of the vaccine candidate. This same approach has been used to design vaccine candidate multi-epitope proteins against other bacterial, viral, and parasitic infections [22-26].

These results reinforce the importance of the use of external membrane proteins for the design of vaccines against infections of gram-negative bacteria as previously reported[9-11]. External membrane proteins are good candidates for vaccine design. This algorithm has proven to be highly sensitive and has been used successfully to predict candidate proteins for a recombinant mortality and morbidity of this disease by reinforcing control measures in Peru. In addition, it would save the funds used to treat cases, both for the health programs and the families of the cases. The use of the attenuated pathogen as a vaccine for this disease would have the risk of reversal of the attenuation. In addition, the preparation of total antigens or fractions is also not viable in this case because it is a bacterium of difficult growth. The use of recombinant antigens is an option that offers the advantage that it is possible to obtain adequate quantities of antigens for the production of a vaccine. Also, a protein chlamydial vaccine. In this study the majority of candidates (74/112) were hypothetical proteins[22], while for *Brucella*, eight hypothetical proteins of 32[23] were selected. This finding is consistent with the fact that about 27% of the proteins that make up the *B. bacilliformis* proteome are hypothetical proteins[27]. In addition, PMOs detected in B. bacilliformis correlate with those previously reported[18]. It should be noted that these results must be confirmed by proteomic analysis of OMP of *B. bacilliformis*.

The multiepitope protein M1 has been designed against infections with *B. bacilliformis*; however, immunization with this protein could generate some degree of protection against infections with other *Bartonella species* in a cross way since the selected epitopes present some degree of conservation with epitopes of other species. Likewise, the methodology described in this article could be useful to design vaccines against infections of other *Bartonella* species of public health importance such as: *Bartonella ancashensis, Bartonella rochalimae, Bartonella henselae, Bartonella quintana, and Bartonella clarridgeiae.*

Among the limitations we have that there is no animal model for Carrion's disease that is useful for the development of a vaccine. That is why we have chosen to use an in vitro infection model. Another plausible model is the infection of monkeys Aotus nancymaae with *B. bacilliformis*[21]; however, this model does not reproduce clinical symptoms as in the natural infection in men. On the other hand, the HLA database of peruvian population currently available is very limited. It is recommended that this database be expanded to make more accurate predictions.

It should be noted that antibodies obtained using alumina as an adjuvant show better results in the invasion assay, this observation can be attributed to the different properties of these adjuvants. Alumina increases the availability of the antigen and the activation of antigen-presenting cells while being a poor inducer of a cell-mediated immune response. On the other hand, the complete adjuvant of Freund reinforced with incomplete adjuvant preferably stimulates an immune response Th2[28,29].

On the other hand, the multiepitope protein M1 is expected to trigger a cellular immune response as

epitopes for HLA type I have been included in its design. However, this must be evaluated experimentally. The results indicate that the designed multiepitope protein M1 could be used as a vaccine against Carrion disease. More studies are recommended to characterize its immunogenic properties and the most suitable conditions for its possible use as a vaccine.

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Supplementary Material: Available in the electronic version of the RPMESP.

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