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ORIGINAL RESEARCH

Bioprospecting by Phage Display of Mimetic Peptides of *Chlamydia trachomatis* for Use in Laboratory Diagnosis

Larissa Silva de Freitas[1,*], Maria Alice Freitas Queiroz[1,*], Luiz Fernando Almeida Machado[1], Antonio Carlos Rosário Vallinoto[1], Marluísa de Oliveira Guimarães Ishak[1], Fabiana de Almeida Araújo Santos[2], Luiz Ricardo Goulart[2,†], Ricardo Ishak[1]

[1]Laboratory of Virology, Biological Sciences Institute, Federal University of Pará, Belém, Pará, Brazil; [2]Laboratory of Nanobiotechnology, Genetics and Biochemistry Institute, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil

*These authors contributed equally to this work
†Luiz Ricardo Goulart passed away on 24/10/2021

Correspondence: Maria Alice Freitas Queiroz, Laboratory of Virology, Biological Sciences Institute, Federal University of Pará, Belém, Pará, Brazil, Tel +55 91 3201-7587, Email alicefarma@hotmail.com

Background: *Chlamydia trachomatis* infection is a major public health problem and the most common sexually transmitted infection in the world. Although highly prevalent, 70% to 80% of cases are asymptomatic and undiagnosed.

Purpose: To overcome some limitations in terms of rapid diagnosis, phage display technology was used to bioprospect peptide mimetics of *C. trachomatis* immunoreactive and immunogenic antigens to be selected for the production of synthetic peptides.

Methods: Initially, IgG from 22 individuals with *C. trachomatis* and 30 negative controls was coupled to G protein magnetic beads. The phage display technique consisted of biopanning, genetic sequencing, bioinformatics analysis and phage ELISA.

Results: Clones G1, H5, C6 and H7 were selected for testing with individual samples positive and negative for *C. trachomatis*. Reactions were statistically significant ($p < 0.05$), with a sensitivity of 90.91, a specificity of 54.55, and AUC values >0.8. One-dimensional analysis with *C. trachomatis* components indicated that the G1 clone aligned with cell wall-associated hydrolase domain-containing protein, the H5 clone aligned with glycerol-3-phosphate acyltransferase PlsX protein, the C6 clone aligned with a transposase and inactivated derivatives, and the H7 clone aligned with GTP-binding protein. Molecular modeling and three-dimensional analysis indicated the best fit of the four clones with a protein known as chlamydial protease/proteasome-like activity factor (CPAF), an important virulence factor of the bacterium.

Conclusion: The peptides produced by phage display are related to the metabolic pathways of *C. trachomatis*, indicating that they can be used to understand the pathogenesis of the infection. Because of their high sensitivity and AUC values, the peptides present considerable potential for use in platforms for screening *C. trachomatis* infections.

Keywords: *C. trachomatis*, phage display, mimetic peptides, CPAF, screening tests

Introduction

Chlamydia trachomatis, a causative agent of serious diseases such as urethritis, venereal lymphogranuloma and trachoma,[1–6] may present in its development cycle structures called elementary bodies (extracellular and infective) and reticular bodies.[7,8] *C. trachomatis* has a trilaminar membrane containing liposaccharides and proteins[9,10] and antigens common to the genus *Chlamydia* and species- and serotype-specific antigens.[11] These antigens include the major outer membrane protein (MOMP), which is related to the pathogenesis of infection and laboratory diagnosis and allows the definition of 19 antigenic variants of the species.[12–17]

The laboratory diagnosis of *C. trachomatis* can be performed by traditional methods ranging from isolation in cell culture to nucleic acid detection; however, technical difficulties remain, but once overcome, the sensitivity and specificity standards

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of diagnostic methods may be improved.[18–20] In Brazil, the gold standard for characterizing the infection is isolation of *C. trachomatis* in cell cultures, a methodology that is rarely used (sensitivity of 70–90%), as it requires specialized personnel, a specific laboratory and strict requirements for collection, transportation and handling of the samples.[21–24]

Direct immunofluorescence is another technique used, but readings of the results under the immunofluorescence microscope are often difficult and subjective. Molecular methods using PCR are sensitive and specific, but the technique is not widely available for small- and medium-size laboratories, as it requires specialized personnel and is costly.[21] Serological methods for antibody detection have been available for many decades, but cross reactivity with other species of *Chlamydia* is common.[25,26] The use of microimmunofluorescence to discriminate serological reactions according to serotypes of *C. trachomatis* and *C. pneumoniae* was a gold standard, but reagents are no longer readily available.[27,28] These are the main reasons why we must seek new, rapid and more precise diagnostic platforms, which are continuously being investigated.[29,30]

The phage display method is a fast, simple, accessible and relatively inexpensive technology that can select high-affinity ligands and define relevant mimetic peptides that can be used in diagnostic assays and vaccine systems.[31–35] The technique is an important low-cost approach for the identification of peptides that can be used in different health areas; the results can be quickly defined and readily used for the diagnosis of several human infectious diseases.[35–39] The identification of mimetic peptides of *C. trachomatis* may be the basis for future methods for rapid diagnosis (point of care), which could be used in medical offices within local infectious disease support clinics, home care testing and screening tests for epidemiological studies, among others.

The objective of this study was to bioprospect, identify and characterize mimetic peptides of *C. trachomatis* antigens for use in serological diagnostic platforms.

Materials and Methods

Sample Collection and Processing

Blood samples from women older than 18 years living in the state of Pará, Brazil, who spontaneously sought cervical cancer prevention services at the Laboratory of Cytopathology, Biological Sciences Institute (Instituto de Ciências Biológicas - ICB) of Federal University of Pará (Universidade Federal do Pará - UFPA) were analyzed. The blood samples were collected into a vacuum collection system containing EDTA as an anticoagulant and then transported to the Laboratory of Virology (ICB-UFPA), where they were centrifuged at 3500 rpm for 15 minutes to separate the plasma and cellular components, which were then frozen at −80 °C until use.

Two commercial immunoenzymatic ELISA tests (SERION ELISA classic) for the detection of antibodies (anti-*Chlamydia trachomatis* IgG and anti-*Chlamydia pneumoniae* IgG) were used to characterize the samples into three groups: 30 samples negative for *Chlamydia* (negative control, NC), 22 samples positive only for *C. trachomatis* (positive control, CT+) and 30 samples positive only for *C. pneumoniae* (CP+). Samples were selected from patients with a cytological diagnosis (Papanicolaou smears) suggestive of *C. trachomatis*, confirmed by PCR, and positive for IgG anti-*C. trachomatis*.

Phage Display

The phage display technique followed a previously described method.[40] First, the IgG antibodies of each group (NC, CP+ and CT+) were bound to magnetic beads coupled with G protein (Dynabeads–Invitrogen). Biopanning consisted of three selection cycles (three rounds), and in each cycle, the phages were subjected to two negative selection steps (NC IgG, CP+ IgG) and one selection step with the target of interest (CT+ IgG). A library of random peptides fused to the N-terminal region of Protein III (pIII) of phage M13 (Ph.D.-C7C; New England BioLabs® Inc.) was added to the NC IgG pool. After incubation at 37 °C, the supernatant was transferred to the tube containing the CP+ IgG pool (both used for negative selection) and subsequently to the CP+ IgG pool (positive selection). The incubation performed at each step lasted 30 minutes. The phages coupled to the antibodies bound to the magnetic microspheres of the positive selections were recovered by acid elution and subjected to amplification, titration, supernatant production, DNA extraction and DNA sequencing.

Phage Amplification

Amplification consisted of phage multiplication and was performed in Luria-Bertani culture medium (LB medium) supplemented with tetracycline and *E. coli* (ER2738 from New England BioLabs® Inc.). The next day, the material was centrifuged at 10,000 rpm for 10 minutes at 4 °C and washed with sterile 1x PBS. Then, the suspension containing phage was incubated in PEG/NaCl, and finally, the isolated phages were suspended in 1x PBS.

Titration

Titration was performed to determine the input and output of viral particles during the biopanning cycles. Nonamplified eluate (diluted from 10^{-1} to 10^{-5}) and amplified eluate (diluted from 10^{-1} to 10^{-12}) were used, with only the last three dilutions of each eluate in the titration being used. *E. coli* ER2738 was grown in LB medium, and upon reaching the mid-log phase (OD_{600}~0.5), dilutions containing the phages were added and cultured on LB agar plates containing tetracycline and IPTG/Xgal (isopropyl-beta-D thiogalactopyranoside/5-bromo-4-chloro-3-indolyl-α-D-galactoside). Plating was performed on Top agar, and the plates were incubated at 37 °C for 24 hours.

Phage Supernatant Production

Phage supernatants were produced by amplifying the phages in LB medium (with tetracycline) containing *E. coli* ER2738 in deepwell plates, which were incubated under stirring on a shaker at 220 rpm in an oven at 37 °C for 4–5 hours. A different clone of phage grown in Petri dishes was added to each well. The phage supernatant was used to back up the clones and extract DNA from the phages.

DNA Extraction

DNA extraction from phages was performed using iodide buffer. The procedure consisted of cell lysis, protein precipitation, DNA precipitation and DNA hydration. Then, the presence of single-stranded DNA was verified in a 0.8% agarose gel containing HydraGreen™ Safe DNA Stain 20,000X (Hydragene, Piscataway, NJ, USA) by visual comparison with standard M13mp18 purified single-stranded DNA (New England Biolabs, Ipswich, Massachusetts, USA).

Sequencing Reaction

For the nucleotide sequencing reaction, template DNA, primer-96 gIII (5'-OH CCC TCA TAG TTA GCG TAA CG-3' - Biolabs) and Premix (DYEnamic ET Dye Terminator Cycle Kit; Amersham Biosciences, Amersham, UK) were used. The reaction was performed in a thermocycler (Kasvi, São José dos Pinhais, PR, Brazil). The sequenced DNA was precipitated and resuspended in dilution buffer (DYEnamic ET Dye Terminator Cycle Kit – Amersham Biosciences) and read in an ABI PRISM™ 3130 Genetic Analyzer (Applied Biosystems, Massachusetts, USA).

Bioinformatics Data Analysis

The DNA sequences were analyzed using bioinformatics programs available online. Translation was performed using ExPASy Translation Tool 6.0 (http://web.expasy.org/translate/), amino acid alignment was performed using BioEdit 7.1.5.0, and the peptides were aligned with *C. trachomatis* proteins (taxid 813) deposited in the NCBI BLAST database. The search for *C. trachomatis* proteins for linear alignment was performed using the protein blast platform through UniProtKB/Swiss-Pro. The Universal Protein Resource (UniProt) database and the Protein Data Bank were used to obtain information on the localization, function and three-dimensional structure of the aligned peptides using the Pepitope Server (http://pepitope.tau.ac.il/index.html).

Phage ELISA

Standardization tests were performed to determine the phage concentration containing the selected peptides and the most appropriate antibody dilution to be used. A microtiter plate (Nunc-Immune™ Plate MaxiSorp™ Surface) was sensitized with 10 µg/well anti-M13 monoclonal antibody (GE Healthcare) diluted in 50 mM carbonate buffer, pH 9.6 (1:500), and incubated overnight at 4 °C. After washing, the plate was blocked with 300 µL of 1x PBS + Molico powdered milk (5%) for 1 hour at 37 °C. The phage clones were diluted in 1x PBS, added to the plate and incubated for 1 hour at 37 °C. The plate was washed, and pools of CT+ and NC plasma were added. After washing, peroxidase-conjugated anti-human IgG

(Sigma/Aldrich) diluted in 1x PBS + 0.1% Tween (1:5000) was added, and the plate was incubated for 1 hour at 37 °C. Finally, TMB was added, and the reaction was stopped by the addition of H_2SO_4 (2 M). The absorbance values were measured using a reading filter at a wavelength of 450 nm. The cutoff value was ≥0.364, with a standard deviation ≥2.

After standardization of the ideal conditions using a plasma pool, another ELISA was performed with individual patient and control samples to verify the reactivity with each selected phage. The method used was the same as that used in the phage ELISA (11 additional different CT+ and NC+ samples).

Statistical Analysis

GraphPad Prism version 6.0 (GraphPad Prism Software Inc., USA) was used to calculate the area under the receiver operating characteristic (ROC) curve (AUC), sensitivity, specificity and positive likelihood ratio (LR+).

Results

Prevalidation with Phage ELISA, Optimization and Reactivity with Individual Samples

Biopanning and sequencing allowed the selection and identification of 25 clones, which were evaluated in a prevalidation using an immunoenzymatic ELISA. The probable mimetics of *C. trachomatis* were selected from clones that showed the highest ratio between the optical density (OD) for the CT+ and CP+ pools and the highest ratio between the OD for the CT+ and NC pools. Clones G1, A5, G5, H5, C6 and H7 were selected for the optimization phase of the test (Figure 1A). Among these clones, those with a CT+/CP+ OD less than 0.364 or CT+/NC OD less than 2 were excluded from the reactivity test with individual samples.

Clones G1, H5, H7 and C6 were selected for the final phage ELISA test, which evaluated the reactivity with individual samples. The reactivity of the samples indicated the same pattern among the different clones evaluated (Figure 1B-E).

Sequencing and Bioinformatics Analysis

The four clones that showed the best performance in the ELISAs were selected for bioinformatics analysis. All of them presented primers and terminators, and the 21 nucleotides corresponded to seven amino acids. After linear alignment of the amino acid sequences of peptides G1, H5, H7 and C6 aligned with amino acid sequences of different *C. trachomatis* proteins (Table 1).

The amino acid sequences were aligned with the proteins deposited in GenBank (NCBI BLAST database) to compare the homology between the sequences obtained and the *C. trachomatis* proteins. The peptides of the evaluated clones were analyzed with regard to the conformational structure of the identified *C. trachomatis* proteins. All the peptides of the clones had sequences that were exposed to several investigated proteins, and the best fit alignment of the four clones was observed on the surface of the protein known as chlamydial protease/proteasome-like activity factor (CPAF; Protein Data Bank accession number: 3DOR; Figure 2).

Area Under the Curve, Sensitivity, Specificity and LRs

Phage ELISA indicated that the four clones presented equal sensitivity and specificity values, a statistically significant p value ($p < 0.05$) and high LR+ values (Figure 3). The sensitivity and specificity values for each clone were plotted on an ROC curve, showing AUCs with satisfactory and equivalent values (Figure 3).

Discussion

Despite the absence of sensitive and specific assays for the serological diagnosis of *C. trachomatis*, the present study is unprecedented because no record of the selection of mimetic peptides for the bacterium is available from commercial peptide libraries using phage display technology.

The amino acid sequences of clones G1, H5, C6 and H7 were aligned with *C. trachomatis* proteins; however, they were not related to MOMPs, proteins usually present as antigens in immunoenzymatic assays for the detection of antibodies. From the UniProt database, the proteins to which the clones aligned were shown to be located in the transmembrane region of the bacterium.

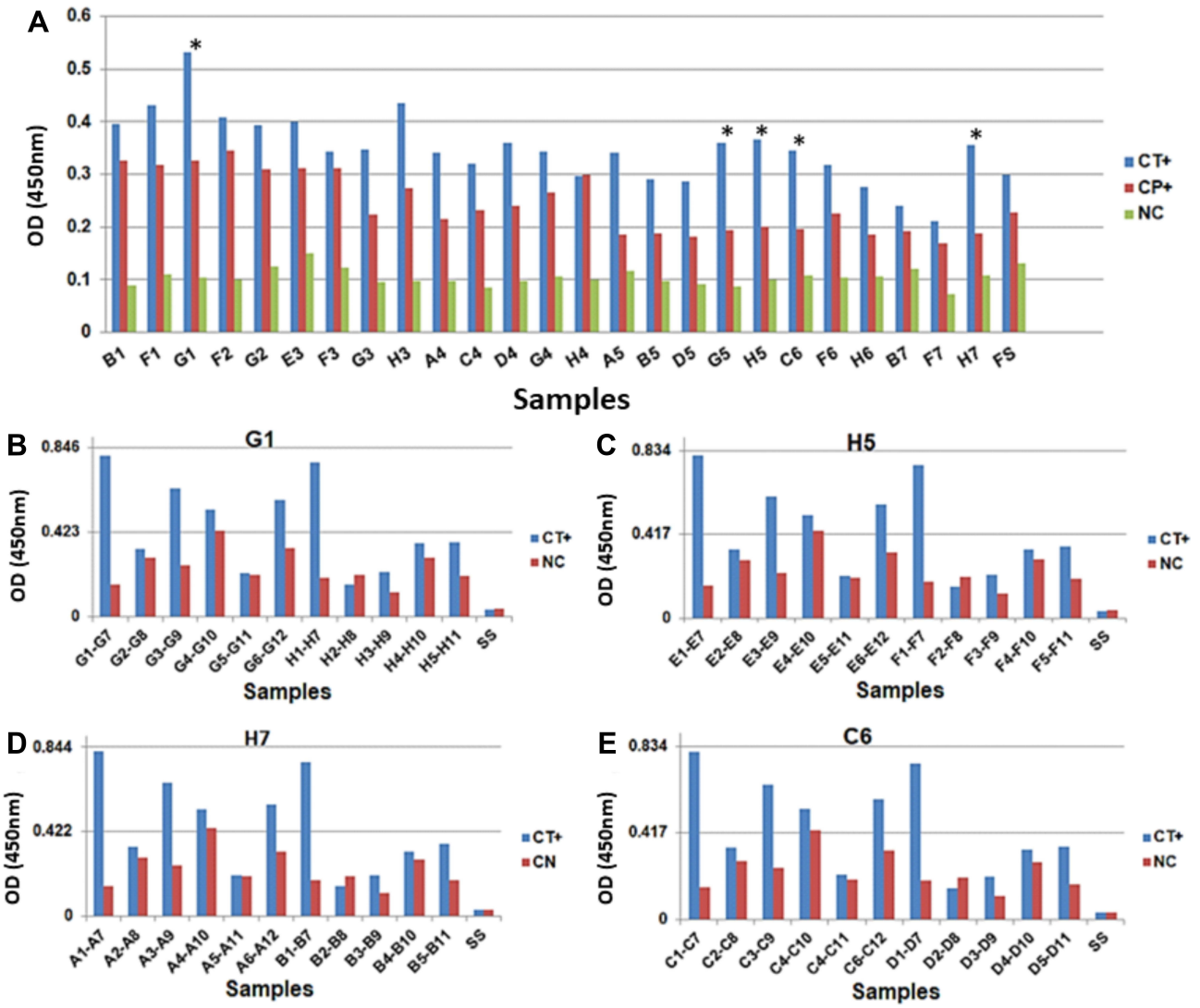

Figure 1 Comparison of the reactivity of clones identified by phage display between the sera of patients who were positive and negative for *C. trachomatis*. (**A**) Phage ELISA with the clones selected by biopanning and sequenced. The clones marked with "*" were selected for use in the ELISA optimization test. Evaluation of reactivity by phage ELISA of individual samples with clones G1 (**B**), H5 (**C**), H7 (**D**) and C6 (**E**).

The peptide from clone G1 (ACMKVSPDSCGGGS) showed similarity with a protein containing a hydrolase domain associated with the cell wall. Hydrolases are ubiquitous enzymes that act on cell division, cell wall rearrangement, bacterial growth and cell lysis. The activity of hydrolases associated with the cell wall may have secondary effects on several physiological functions, such as bacterial adhesion, biofilm formation, protein secretion, conjugation, virulence and immune responses.[27,28,41,42]

The peptide from clone H5 (ACNHARTLTCGGGS) aligned with the protein glycerol-3-phosphate acyltransferase, which acts in the biosynthetic pathway for the formation of phosphatidic acid in the biosynthesis of bacterial membrane phospholipids, whose main function is maintaining plasma membrane structure.[43,44] Other proteins have been observed in metabolic model studies, providing details about the metabolic adaptations of *C. trachomatis* during infection, which helps in understanding important details of the biology of *Chlamydia* infection.[45]

The peptide from clone C6 (ACYHHDSRGCGGGS) aligned with transposase enzymes and inactivated derivatives. Transposases recognize the start and end points of a transposon, catalyzing the transposition to another gene region. These transposition elements and the associated transposase genes are the most abundant and ubiquitous in nature and are

Table 1 Amino Acid Sequences of the Clones That Showed the Best Performance in the Phage ELISA Tests

Clone	Peptide	Alignment	Query cover	%Id	Putative target	GenBank
G1	AC**MKVSPDS**CGGGS	SPDSCGGGS SPDS G GS SPDS-GSGS	74%	77.7%	Cell wall-associated hydrolase domain-containing protein	CRH69585.1
H5	AC**NHARTLT**CGGGS	HART–LTC HART L C HARTQPLVC	71%	66.6%	Glycerol-3-phosphate acyltransferase PlsX	CRH67789.1
C6	AC**YHHDSRG**CGGGS	YHHD-SRGC YHHD SR C YHHDPSRSC	57%	77.7%	Transposase and inactivated derivatives	CPR81983.1
H7	AC**ALPSDRT**CGGGS	LPSDR LPSDR LPSDR	64%	100%	GTP-binding protein	CRH84351.1

Abbreviation: Id, identification.

essential for adaptation and biological diversity.[46] Through mutagenesis studies, sets of genes essential for *C. trachomatis* may be able to be identified to gain a better understanding of the biology of bacterial infection, leading to the discovery of new drug or vaccine targets.[47]

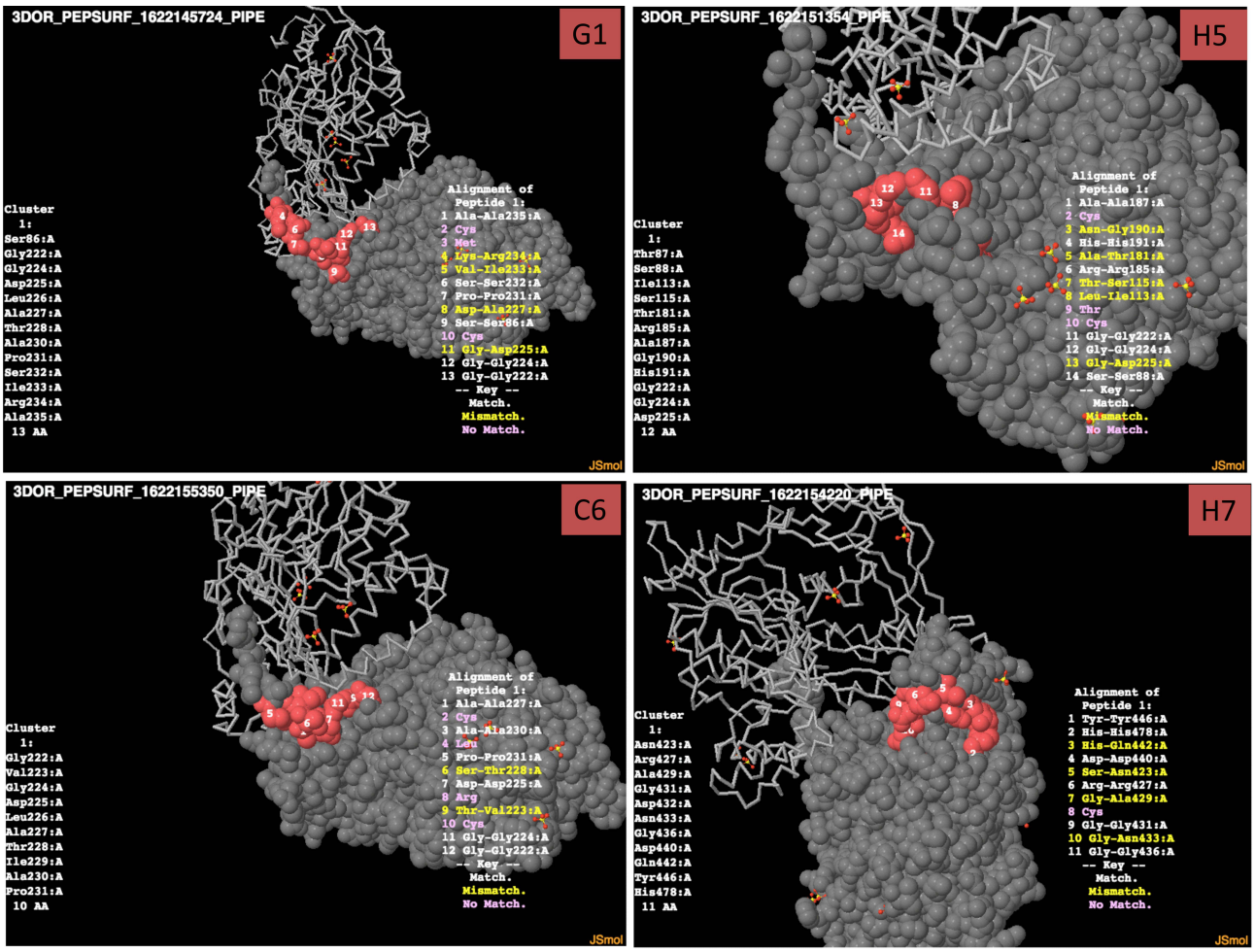

Figure 2 Conformational alignment of peptides from clones G1, H5, C6 and H7 in CPAF of *C. trachomatis*.

Clone	Sensitivity (95% CI)	Specificity (95% CI)	AUC	*cut-off*	*p* value	LR+
G1	90.91 (58.72%-99.77%)	54.55 (23.38%-83.25%)	0.8058	> 0.2140	0.01515	2.000
H5	90.91 (58.72%-99.77%)	54.55 (23.38%-83.25%)	0.8099	> 0.2070	0.01383	2.000
C6	90.91 (58.72%-99.77%)	54.55 (23.38%-83.25%)	0.8099	> 0.2050	0.01383	2.000
G1	90.91 (58.72%-99.77%)	54.55 (23.38%-83.25%)	0.8058	> 0.2140	0.01515	2.000

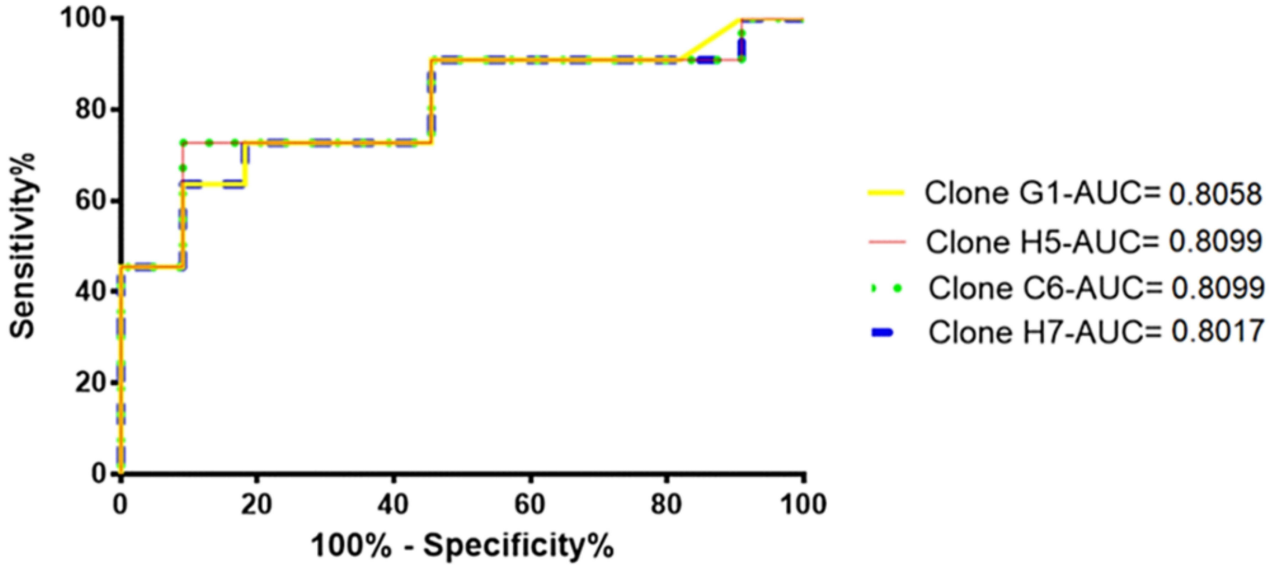

Figure 3 Statistical parameters of the mimetic peptides. Sensitivity, specificity, likelihood ratio (LR) and area under the curve (AUC) and ROC curve.

Clone H7 (ACALPSDRTCGGGS) aligned with a GTP-binding protein. GTP is related to the modulation of host signaling pathways and may play an important role in the pathogenesis of *Chlamydia*, comparable to the activities of MOMP.[48] This mimetic peptide can be used in assays to describe new signaling pathways of components involved in the pathogenesis of *Chlamydia* infection.

The three-dimensional alignment of the clones with different proteins of *C. trachomatis* revealed CPAF as the best match.[49,50] This decision was reached considering that the structure of CPAF accommodated all four clones in contrast to the other proteins for which maximum matches ranged from 1 to 3. The pathogenic mechanism of infection and the diseases associated with *Chlamydia* infection result from the onset of an inflammatory process, which is not well understood, and to survive in the host, *Chlamydia* must evade the host's immune response. CPAF has different and important functions in the evasion of the immune response by mechanisms that may include cleaving or degrading host proteins and acting as a "strong virulence factor" during infection.[50–54] The absence of CPAF is crucial in the multiplication of *Chlamydia* and implies the absence of the formation of infectious elementary bodies.[53] Importantly, CPAF is highly conserved in *Chlamydia* species, both regarding its structural composition and its functionality, eg, when examining a similar protease activation mechanism in different *Chlamydia* species.[55]

The peptides of clones G1, H5, C6 and H7 aligned with sequences located on the surface of CPAF; thus, the clones showed a promising immunogenic role that should be investigated, either individually or on a multiplex basis. The selection of these clones seems to suggest that MOMPs are not the only important proteins for the diagnosis of *C. trachomatis* infection. As a novel suggestion, for the first time, proteins involved in the metabolic processes of the bacterium also have diagnostic potential and should be investigated. The targeting of large quantities of antibodies to CPAF, especially the epitopes defined by clones G1, H5, C6 and H7 can detect the presence of active infections and possibly the persistence of the bacterium considering the functions associated with CPAF identified to date.

However, the reactivity of the mimetic peptides obtained with serum samples positive for *C. trachomatis* IgG revealed that the four clones showed high sensitivity (90.91%) and low specificity (54.55%). The immunoenzymatic

assay resulted in LR values for the clones indicative of a twofold greater likelihood of detecting a positive result among infected compared to noninfected samples. In addition, the clones showed AUC values above 0.8, indicating the high discriminative power of the test between the disease and health states. From a clinical perspective, a screening test that can be used as a point-of-care test is crucial to initiate immediate treatment for *C. trachomatis* infections.

Other examples have been described for viral infectious agents (low antigenic complexity), such as hepatitis E virus,[56] bacterial infectious agents, such as *M. tuberculosis*,[57] and those with greater antigenic complexity, such as *Strongyloides*.[58] The change in focus in the selection of traditional antigens indicates good results for the synthesis and selection of mimetic peptides, whose results reach values better than the typical methods using structural and functional/metabolic proteins, such as those in the present study.

The phage display method was used to evaluate *C. trachomatis* infection using single-chain variable fragment (scFv) antibodies presented by phages to analyze the complexity of the surface molecules of the elementary body of *Chlamydia*; this approach allowed the detection and identification of a variety of elementary body-associated antigens and host cell antigens.[59] Thus, the technique was demonstrated to be important for evaluating infection using different approaches because it allowed the description of relevant peptides, such as those described in the present study, that can be explored in future assays not only for laboratory diagnosis but also for understanding the pathogenesis and treatment of diseases. The selection of clones indicated peptides different from those that are usually selected as antigens in assays for the detection of antibodies against *C. trachomatis*. Notably, the selection of antigens for diagnostic use is more difficult in regard to the antigenic complexity of bacteria. The opportunity to select the four peptides is novel in that they relate to a protein of the metabolic pathway of *C. trachomatis* that differs from what has been used to date. In contrast, due to the innovation involved, data with which to compare the results obtained are lacking; thus, the peptides can be inferred to have sufficient relevance for future evaluation for the purpose of diagnostic screening for *C. trachomatis* infection. The approach used to select different epitopes in different proteins of the infectious agent has also been successfully attempted using other selection methods for HTLV as a novel opportunity for diagnosis.[60]

In the present study, the phage display method showed the importance of using CPAF for diagnosis, but this protein has already been used in a murine model as a sufficient immunizer against *C. muridarum*, inducing protective $CD4^+$ T lymphocyte-dependent immunity and IFN-γ production.[61] The description of clones G1, H5, C6 and H7 and the conserved structure of CPAF indicate the presence of four epitopes that are also conserved, a finding that can be tested for the production of future vaccines with universal use for human primates and other animals infected by *Chlamydia* species.

In conclusion, the use of the phage display methodology allowed bioprospection of mimetic peptides of *C. trachomatis*. Four phage clones were associated with the metabolism of the bacterium, which are promising peptides to be used in diagnostic platforms using immunochromatography or biosensors for an efficient, rapid, easy-to-perform and low-cost test.

Ethics Approval and Informed Consent

The present study was approved by the Ethics Committee for Research with Human Beings of Fundação Hemopa (opinion 0011.0324.000-1) in accordance with the Declaration of Helsinki. The participants were informed about the objectives of the study, and those who agreed to participate signed an informed consent form.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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