Theranostic applications of phage display to control leishmaniasis: selection of biomarkers for serodiagnostics, vaccination, and immunotherapy


INTRODUCTION

Leishmaniasis is a group of cutaneous and visceral infections caused by protozoan parasites belonging to the genus *Leishmania*[1]. The disease is characterized by high morbidity and mortality; it is spread in 98 countries and three continents (Asia, Africa, South and Central America), where 350 million people are at risk of contracting *Leishmania* infection[2][3]. Canine visceral leishmaniasis (CVL) caused by *Leishmania* (*Leishmania* infantum *chagasi* is a major global zoonosis potentially fatal to humans and dogs. The infection is considered endemic in approximately 70 countries of southern Europe, Africa, Asia, and Central and South America[3][4]. However, geographic distribution of CVL is expanding throughout the Western hemisphere, and the disease can be currently found in countries from Argentina to the United States[5], reaching as far as Southern Canada[6].

Historically, leishmaniasis has been treated by chemotherapy using pentavalent antimony compounds like meglumine antimonate and stibogluconate. However, these drugs can be clinically ineffective in some visceral leishmaniasis (VL) cases which tend to relapse at a later stage[7][8], and may cause side effects such as myalgias, arthralgias, pancreatitis, leucopenia, and renal, hepatic, and cardiac toxicity[9]. The World Health Organization (WHO) has recommended the use of liposomal ampicillin B (L-AmpB) because of its efficacy and safety[10]; however, despite the improvement in therapeutic indexes shown by L-AmpB, its application remains limited, mainly because of the high cost[11].

In their life cycle, *Leishmania* parasites progress through several stages in different hosts and have developed sophisticated mechanisms for host invasion and immune escape. The first step in the interaction between the host and the parasite is the recognition and binding to host cell receptors. *Leishmania* spp. species express a variety of surface and secreted molecules used by the parasite to attach and enter mammalian cells. These factors are key determinants of the disease progression, and most studies on host-pathogen interactions are focused on the identification of *Leishmania* ligands and related host receptors using classical biochemical approaches such as affinity purification, cross-linking, immunoprecipitation, and fractionation[12]. However,
these techniques are not intended for high-throughput screening of multiple candidate molecules. One powerful approach that allows overcoming this limitation in the discovery of new parasite antigens is phage display technology(13). Phage display is a subtractive proteomic technique based on the cloning of foreign deoxyribonucleic acid (DNA) in a filamentous phage and presentation of recombinant peptide variants fused to phage outer surface proteins. The method was first described by George Smith in 1985 when he reported the expression of a foreign polypeptide on the surface of phage particles(14). The nucleotide coding sequence of the foreign peptide was genetically fused in frame to the gene encoding bacteriophage coat protein, resulting in a protein hybrid, which presented the target peptide on the outer surface of viral particles(14).

The technique was successfully applied to the construction of bacteriophage libraries displaying multiple random surface peptides, enabling in vitro and in vivo selection and identification of peptide motifs responsible for protein-protein, protein-DNA, enzyme-substrate, and other types of molecular interactions(15)(16)(17). Phage libraries expressing exogenous peptides have been used in the identification of cellular receptors and foreign antigens, antibody epitope mapping, drug discovery, protein engineering, and other applications based on high-affinity interactions between the target and recombinant peptides without prior knowledge of the motifs in question(18). Synthetic sequences mimicking target epitopes can be obtained by screening phage libraries; these short peptides called mimotopes that can be characterized as continuous/linear or discontinuous/conformational epitopes of the proteins’ determinant regions, which may mismatch or only partially match their primary structure, but can perfectly well reproduce its three-dimensional conformation(19).

The selection of phage-displayed molecules with high affinity to a particular target is performed by successive selection steps called biopanning (Figure 1). The target is immobilized on a solid support, and the phage display library in solution is applied to allow binding of specific variants to the target. The number of biopanning cycles depends on the degree of enrichment of phage particles displaying motifs specific for the immobilized target. Usually, three to five biopanning cycles are required for a population of phage clones with high affinity to the target; implementation of more than five cycles

![FIGURE 1 - Biopanning cycles in phage display-based antigen selection. Phage particles displaying antigens with high affinity to the immobilized target molecule can be recovered using different elution protocols, including acid elution (glycine•HCl, pH 2.0) or competition for the binding to the immobilized target. Recovered phage clones are amplified, titrated, and sequenced.](image-url)
can favor the selection of high-affinity variants within the viral population, which outcompete those with low affinity, thereby negatively affecting clonal diversity\(^{20}\). This review explores potential use of phage display technology for theranostics of leishmaniasis, focusing on recent improvements in biomarker discovery strategies that have led to the identification of novel vaccine candidates and diagnostic markers for VL.

**BACTERIOPHAGES AS TOOLS FOR THE DEVELOPMENT OF NEW VACCINES, DIAGNOSTIC MARKERS, AND DRUGS**

During recent decades, phage display has been widely used in medicine and biotechnology, promoting the discovery of new drugs and vaccine candidates, and the improvement of diagnostic tools for various diseases\(^{21}\). Thus, it has been successfully applied to identify mimotopes used to diagnose malaria\(^{22} (23)\), toxoplasmosis\(^{24} (25)\), hepatitis A\(^{26}\), neurocysticercosis\(^{27}\), strongyloidiasis\(^{28}\), thyroid cancer\(^{29}\), Chagas’ disease\(^{30}\), and bovine anaplasmosis\(^{31}\), and to develop vaccine candidates against cysticercosis\(^{32}\), herpes simplex virus infection\(^{33}\), cancer\(^{34}\), taeniasis\(^{35}\), hepatitis B\(^{36}\), trichinellosis\(^{37}\), Alzheimer’s disease\(^{38}\), and bovine anaplasmosis\(^{39}\). Phage display technology has been also instrumental in the selection of therapeutic agents to treat various cancers such as glioblastoma, melanoma, leukemia, and prostate and thyroid cancers\(^{40} (41)\).

Several studies have applied phage display for the development of disease diagnostic markers. The strategy was first used in neurocysticercosis diagnostics\(^{42}\). Recently, a phage-based enzyme-linked immunosorbent assay (ELISA) assay has been employed to evaluate transmissible gastroenteritis virus infection in pigs\(^{43}\), the study showed that ELISA coupled with phage display was a more sensitive method than conventional antibody-based ELISA. In another study, three peptides expressed in reactive phage clones and selected against serum from leprosy patients were successfully validated as tools for serodiagnostic of leprosy\(^{44}\). A similar approach has been applied to develop diagnostics for other animal and human diseases, such as neurocysticercosis\(^{27}\), strongyloidiasis\(^{28}\), and bovine anaplasmosis\(^{39}\).

Phage particles carrying antigenic determinants may be directly used for therapy. Thus, phage display was applied to select mimotopes for the treatment of *Mycobacterium ulcerans* in a mouse model. The authors have demonstrated that a single subcutaneous injection of a specific (D29) mimotope-expressing bacteriophage administered 33 days after bacterial challenge was effective in reducing the infection and preventing ulceration.

The protection resulted in a significant reduction of bacterial burden accompanied by increased production of cytokines, including interferon-gamma (IFN-γ), both in the infected footpads and draining lymph nodes. The treatment with D29 mimotope also stimulated the increase in infiltrating lymphocytes and macrophages. The study has demonstrated a potential of phage-based therapy against *M. ulcerans* infection, paving the way for the development of novel phage-based therapeutic approaches\(^{45}\).

Phage-displayed peptides employed as vaccine candidates have two important advantages. First, bacteriophages presenting antigenic and immunogenic determinants can be taken up by phagocytic cells and processed efficiently, enabling peptide presentation by major class I and II histocompatibility complexes\(^{46} (47)\). Second, the amplification of peptides expressed on phage particles is easier and less expensive compared to conventional chemical synthesis or recombinant protein expression. Moreover, the final product consists of multiple virus copies providing high level of mimotope exposure to the host’s immune system. In addition, bacteriophages are not pathogenic to humans and can replicate inside phagocytic cells\(^{37} (48)\). An important aspect of using phage peptide clones as vaccine candidates is the immunostimulatory effect of non-methylated cytosine-phosphate-guanosine (CpG) motifs present in phage genome, which can contribute to the activation of the mammalian immune system through Toll-like receptors\(^{49} (50)\). Thus, the application of peptide-carrying bacteriophages can reduce or eliminate the need for adjuvants, which are administered together with synthetic peptides and recombinant proteins to activate and/or improve immunological response to vaccine candidates\(^{51}\).

**PHAGE DISPLAY OF DUAL-FUNCTION PEPTIDES TO CONTROL LEISHMANIASIS: A THERANOSTIC APPROACH**

Serological tests are currently recommended for the laboratory diagnosis of CVL. However, the serodiagnostic performance of these tests is hampered by insufficient sensitivity and/or specificity, leading to the occurrence of false-positive results due to cross-reactivity with the antibodies against other parasites, such as *Trypanosoma cruzi*\(^{52} (53) (54)\), *Babesia canis*, or *Ehrlichia chaffeensis*\(^{55}\), or false-negative results in infected animals with low serum levels of antileishmanial antibodies\(^{56} (57) (58) (59)\). Moreover, the two anti-CVL vaccines commercially available in Brazil can induce high production of *Leishmania*-specific antibodies in vaccinated animals, which then can be diagnosed as infected by serological assays\(^{60}\).

In an attempt to identify more refined antigens for the improvement of sensitivity and specificity of CVL serodiagnostics, Costa et al., in 2014\(^{61}\), have employed the sequential subtractive selection of phage-displayed peptides using immunoglobulin G (IgG) antibodies purified from non-infected or those *T. cruzi*-infected dogs and from symptomatic and asymptomatic VL animals. In that study, negative selection was applied to eliminate clones with the affinity to antibodies from non-infected or *T. cruzi*-infected dogs, as well as from animals immunized with Leishmune\(^{6} \) or Leish-Tec\(^{6}\) vaccines\(^{62}\).

The evidence of life-long immunity against *Leishmania* spp. infection has inspired the development of prophylactic...
vaccination models of leishmaniasis, but few of them have progressed beyond the experimental stage\(^{(62)}\) \(^{(63)}\) \(^{(64)}\) \(^{(65)}\) \(^{(66)}\) \(^{(67)}\) \(^{(68)}\). There is evidence that type-1 cell-mediated immunity is important for protective response against VL\(^{(69)}\). Based on the experimental models, several candidates for *Leishmania* vaccine have been identified, including whole parasites\(^{(70)}\), parasite fractions\(^{(71)}\) \(^{(72)}\), recombinant proteins\(^{(73)}\) \(^{(74)}\), polyproteins\(^{(75)}\), DNA\(^{(76)}\) \(^{(77)}\), and synthetic peptides\(^{(78)}\) \(^{(79)}\), which exerted immunostimulatory effects and induced variable degrees of protection against *Leishmania* spp. infection. In search of vaccine candidates against VL, a recent study has used phage display to select parasite-specific immunogens, which were tested in BALB/c mice for their potential to protect against *L. infantum* infection. Phage clones were tested *in vitro* for their selectivity and specificity to induce the production of IFN-γ and interleukin-4 (IL-4), the cytokines characteristic for immune response against *Leishmania* parasites, and two clones, B10 and C01, have been selected (Figure 3). The phage clones were further tested in vaccination protocols together with saponin as an adjuvant, and demonstrated the induction of a T helper 1 (Th1)-specific response in vaccinated animals, which was characterized by the production of IFN-γ, IL-12, and granulocyte-macrophage colony-stimulating factor (GM-CSF), and reduction of IL-4 and IL-10, as well as the predominance of parasite-specific IgG2a antibodies, all of them evaluated by ELISA procedures. B10- and C01-immunized *Leishmania*-
infected mice demonstrated significant reduction in parasite burden in the liver, spleen, bone marrow, and draining lymph nodes compared to controls, including wild-type and non-relevant mimotope-displaying phages (Figure 4), which correlated with higher IFN-γ production by spleen cells of these animals\(^{67}\).

Aiming to develop a protective vaccine that will be able to induce a heterologous protection on leishmaniasis, both clones selected in \textit{L. infantum} were evaluated as immunogens in \textit{L. amazonensis}. This species can cause a wide spectrum of clinical symptoms characteristic for leishmaniasis\(^{79}\). Both clones have elicited protective response against parasite infection in mice, as evidenced by significant reduction of footpad swelling and parasite burden in the infected footpads, liver, spleen, bone marrow, and draining lymph nodes compared to all control groups (Figure 5). The protection was correlated with IFN-γ production mediated by cluster of differentiation 8\(^+\) T cell-specific response to parasite proteins. The protected animals also presented low levels of leishmaniasis-associated IL-4 and IL-10, as well as increased levels of parasite-specific IgG2a antibodies (manuscript in preparation). One important aspect in this study was that phage clones were administered without adjuvants, demonstrating the immunostimulatory activity of phage particles. Additional studies are in developing, aiming to identify the native proteins in \textit{Leishmania} spp. that express these target peptides.

The limitation of most studies related to the development and selection of vaccine candidates for \textit{Leishmania} spp. is the pre-clinical model chosen for initial screening of promising molecules. Although sand fly-transmitted infection in

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**FIGURE 3** - Phage display technology applied to the selection of candidate antigens for the development of \textit{Leishmania infantum} vaccine. Antibodies (IgG) purified from non-infected and \textit{L. infantum}-infected dogs without or with symptoms of visceral leishmaniasis were coupled to magnetic microspheres (beads) conjugated to protein G (A). The IgG-containing microspheres were used for successive biopanning cycles to select phage clones with the affinity to \textit{L. infantum}-specific antibodies purified from parasite-infected dogs (B). Selected clones were used for \textit{in vitro} stimulation of spleen cells derived from naive and chronically infected mice, and the levels of IFN-gamma and IL-4 production were determined. Clone specificity and selectivity were evaluated by comparing IFN-gamma and IL-4 levels in spleen cells stimulated with selected clones with those stimulated with the wild-type phage or a non-relevant phage, respectively. Two phage clones, namely B10 and C01, which showed the best specificity and selectivity values, were selected and used in the vaccination experiments (C). CVL: canine visceral leishmaniasis; IgG: immunoglobulin G; VL: visceral leishmaniasis; IFN-γ interferon gamma; IL-4: interleukin-4; \textit{L.}: \textit{Leishmania}. 
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FIGURE 4 - Vaccination with candidate phage clones protects BALB/c mice against Leishmania infantum infection. BALB/c mice were inoculated with saline or immunized with saponin (25µg, Quillaja saponaria bark saponin; Sigma-Aldrich) or with the bacteriophages (1 × 10^{11} phage particles) plus saponin. Three doses were administered at 2-week intervals; 4 weeks after the last immunization, animals were subcutaneously infected with 1 × 10^7 stationary-phase promastigotes of Leishmania infantum. Parasite load in the liver (A), spleen (B), paw draining lymph nodes (C), and bone marrow (D) was measured 10 weeks post-infection using a limiting dilution method. The data are presented as the mean ± standard deviation of the experimental groups. Statistically significant differences in parasite load between B10- and C01-immunized mice and control (wild-type phage-WTP, saponin and saline groups) mice were found (Costa et al., 2014). ***P < 0.0001. WTP: wild type phage.

Phage display can be considered a robust, accurate, and versatile approach that allows the identification of disease-specific dual-function antigens for both diagnostic and therapeutic purposes. This technique has been successfully applied for the selection of theranostic antigens specific for leishmaniasis. The search for new theranostic biomarkers for diagnostics, vaccination, and/or immunotherapy can be successfully accomplished using phage display methodology, which opens new opportunities in the fight against human and animal diseases.
FIGURE 5 - Vaccination with candidate phage clones protects BALB/c mice against *Leishmania amazonensis* infection. BALB/c mice were inoculated with saline or immunized with bacteriophages (1 × 10^{11} phage particles): WTP, wild-type phage; NRP, non-relevant phage; B10 and C01, selected parasite-specific phage clones; B10/C01, combination of phage clones (5 × 10^{10} particles each) with or without 25 μg of saponin. Three doses were administered at 2-week intervals; 4 weeks after the last immunization, animals were subcutaneously infected with 1 × 10^6 stationary-phase promastigotes of *Leishmania amazonensis*. The course of the disease was monitored weekly and expressed as the increase in thickness of the infected footpad compared to the uninfected footpad (A). Parasite load in the infected footpads was measured 10 weeks post-infection using a limiting dilution method. The data are presented as the mean ± standard deviation; a, b, and c indicate statistically significant differences with the saline group, WTP group, and NRP group, respectively (P < 0.001) (B) (manuscript in preparation). WTP: wild type phage; NRP: non-relevant phage.
CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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