Review Article

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

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ABSTRACT

Phage display is a high-throughput subtractive proteomic technology used for the generation and screening of large peptide and antibody libraries. It is based on the selection of phage-fused surface-exposed peptides that recognize specific ligands and demonstrate desired functionality for diagnostic and therapeutic purposes. Phage display has provided unmatched tools for controlling viral, bacterial, fungal, and parasitic infections, and allowed identification of new therapeutic targets to treat cancer, metabolic diseases, and other chronic conditions. This review presents recent advancements in serodiagnostics and prevention of leishmaniasis -an important tropical parasitic disease- achieved using phage display for the identification of novel antigens with improved sensitivity and specificity. Our focus is on theranostics of visceral leishmaniasis with the aim to develop biomarker candidates exhibiting both diagnostic and therapeutic potential to fight this important, yet neglected, tropical disease.

Keywords: Phage display. Mimotopes. Visceral leishmaniasis. Vaccine. Serodiagnosis.

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].

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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

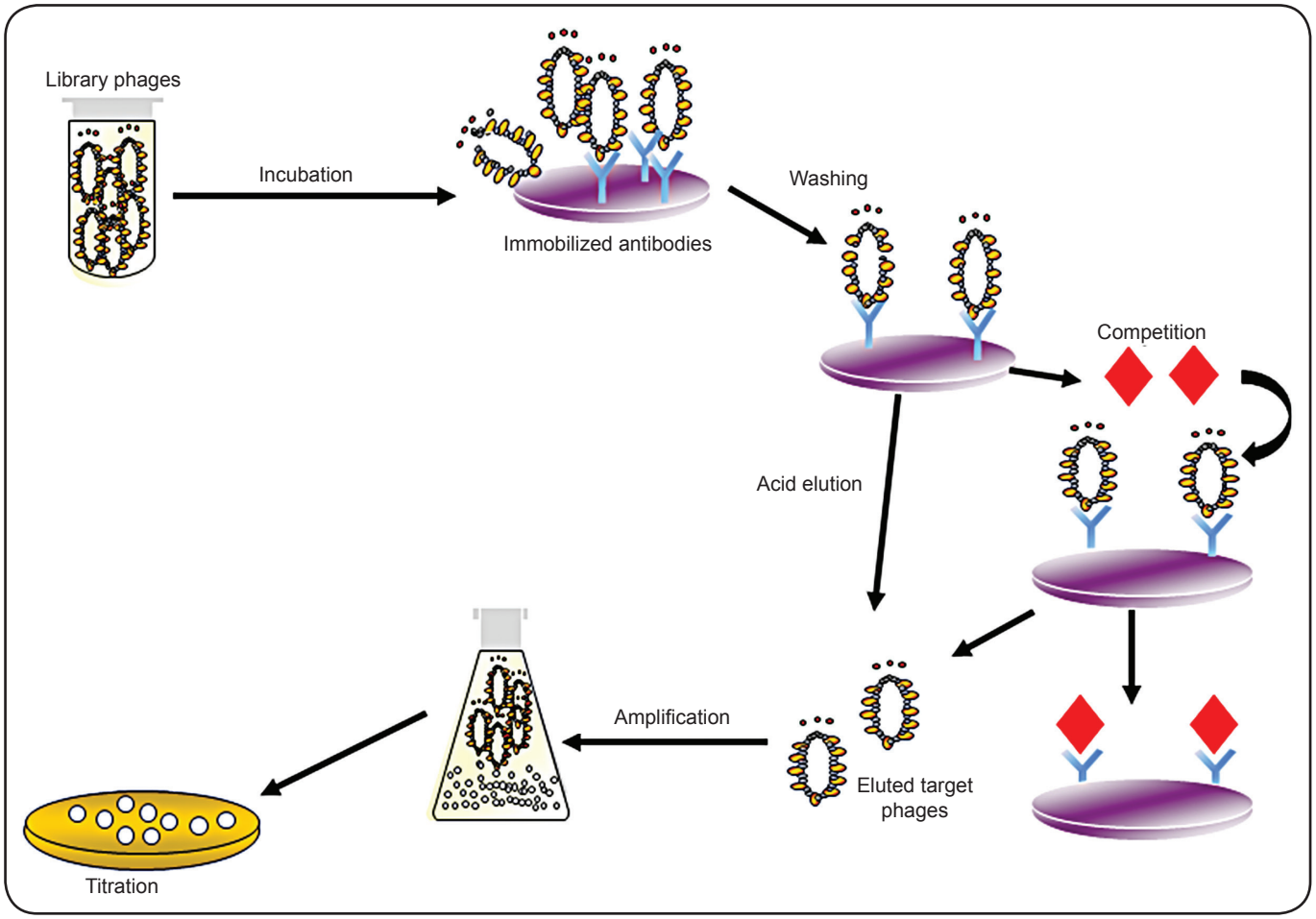

FIGURE 1 - Biopanning cycles in phage display-based antigen selection. Phage particles displaying antigens with high affinity to the immobilized target molecule can be recovered using different elution protocols, including acid elution (glycine•HCl, pH 2.0) or competition for the binding to the immobilized target. Recovered phage clones are amplified, titrated, and sequenced.

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 serological diagnosis 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 canis*[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 and avoid cross-reactivity. The remaining phage particles were then subjected to positive selection using antibodies from asymptomatic and symptomatic *L. infantum*-infected dogs **(Figure 2)** and high-affinity clones were further validated by ELISA. As a result, eight bacteriophage-fused peptides with 100% sensitivity and specificity have been identified. Moreover, no false-positive results were observed based on operating characteristic (ROC) curves calculated for sera from *T. cruzi*-infected or *E. canis*-infected dogs, as well as from animals immunized with Leishmune® or Leish-Tec® vaccines[61].

The evidence of life-long immunity against *Leishmania* spp. infection has inspired the development of prophylactic

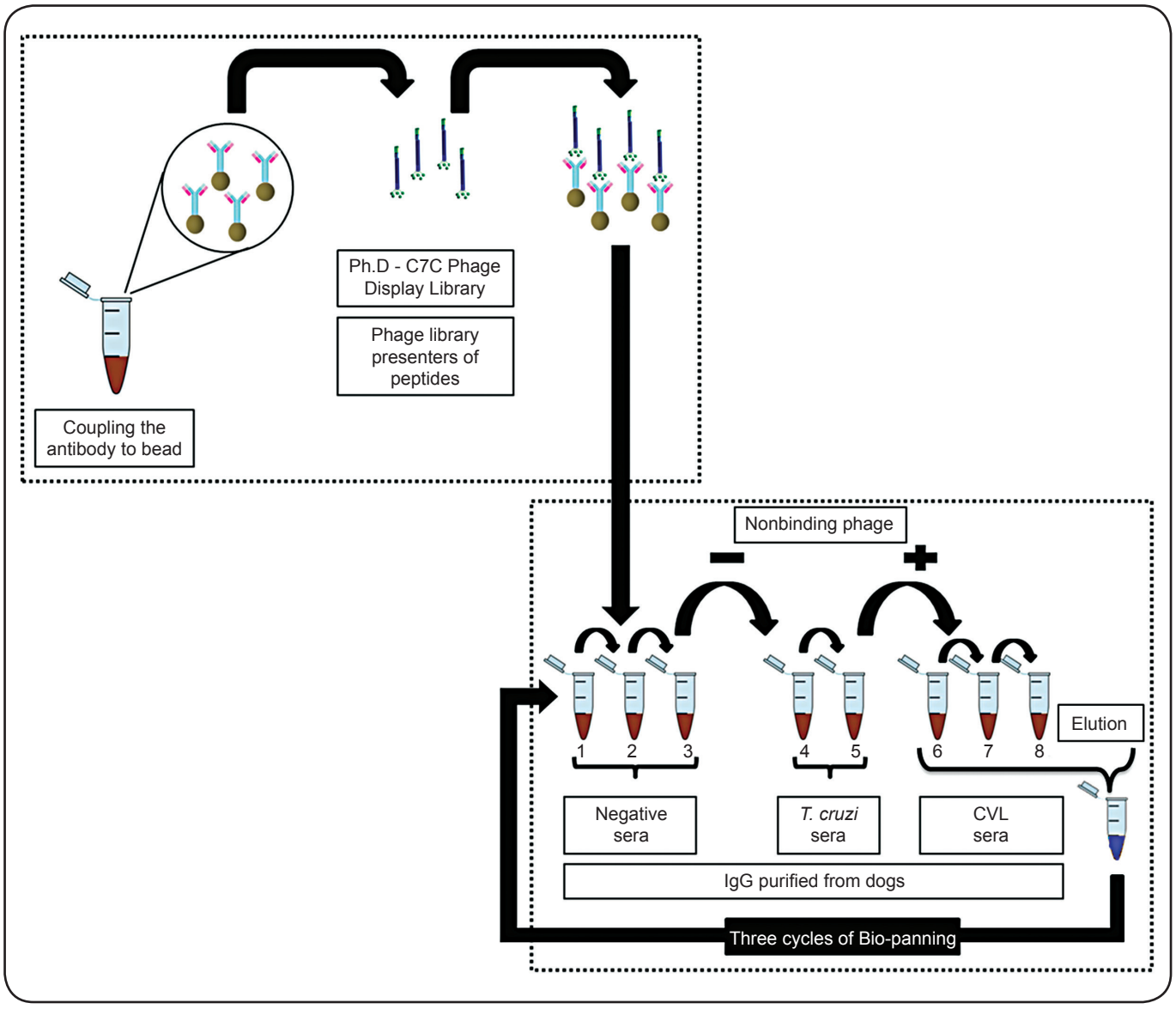

FIGURE 2 - Schematic representation of subtractive selection process in phage display. First, negative selection was performed to reduce non-specific cross-reactivity by removing phage clones with the affinity to unrelated antibodies derived from non-infected or *Trypanosoma cruzi*-infected dogs. Then, the remaining phage particles were subjected to positive selection using specific antibodies from *Leishmania infantum*-infected dogs. Phage clones with the affinity to *Leishmania infantum*-specific antibodies were selected, sequenced, and identified (Costa et al., 2013). *T.*: *Trypanosoma*; **CVL:** canine visceral leishmaniasis; **IgG:** immunoglobulin G.

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], poly-proteins[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*-

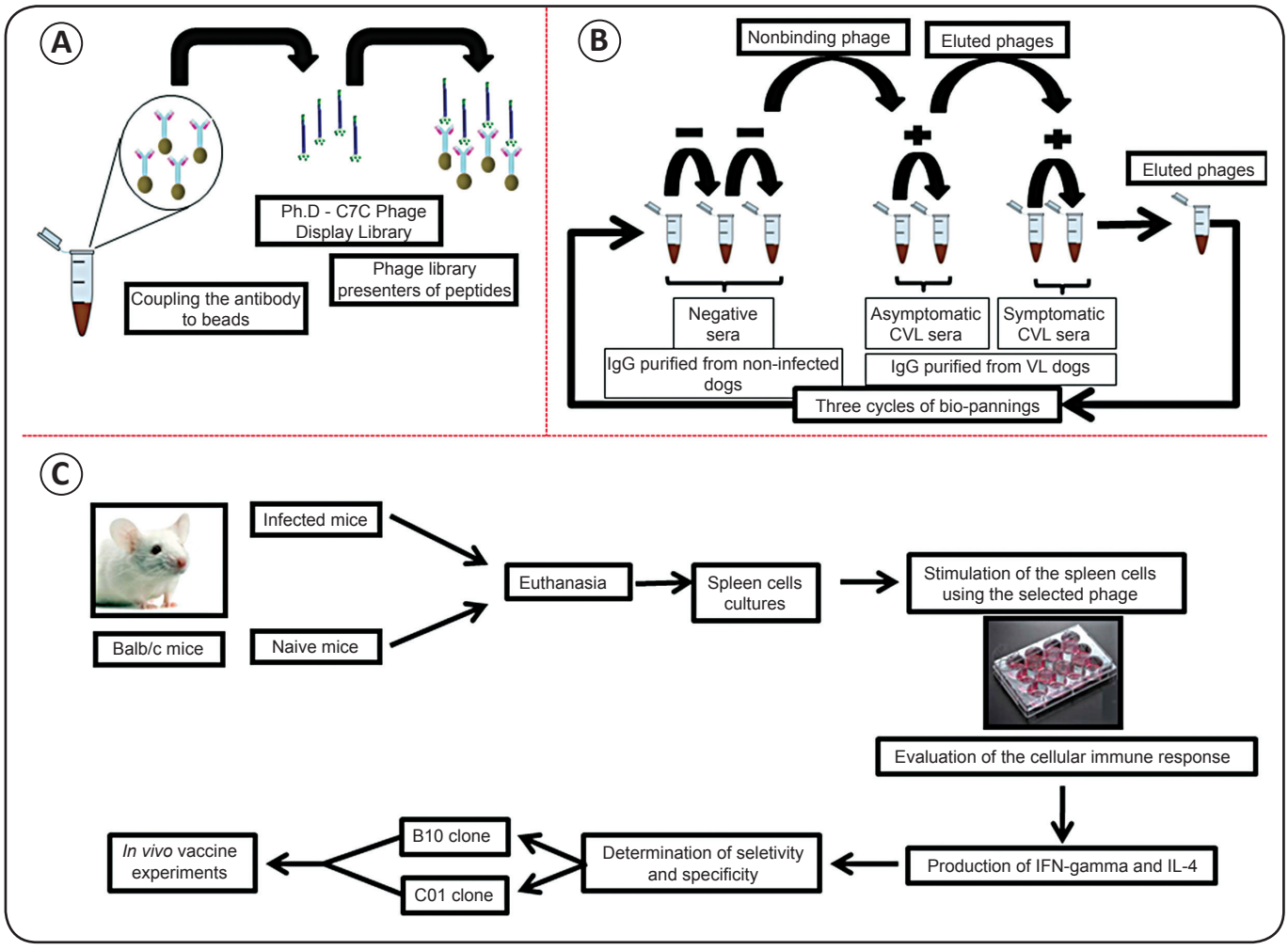

FIGURE 3 - Phage display technology applied to the selection of candidate antigens for the development of *Leishmania infantum* vaccine. Antibodies (IgG) purified from non-infected and *L. infantum*-infected dogs without or with symptoms of visceral leishmaniasis were coupled to magnetic microspheres (beads) conjugated to protein G (A). The IgG-containing microspheres were used for successive biopanning cycles to select phage clones with the affinity to *L. infantum*- specific antibodies purified from parasite-infected dogs (B). Selected clones were used for *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; ***L.:*** *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 *L. infantum* were evaluated as immunogens in *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^+ $(CD8)^+$ 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 *Leishmania* spp. that express these target peptides.

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

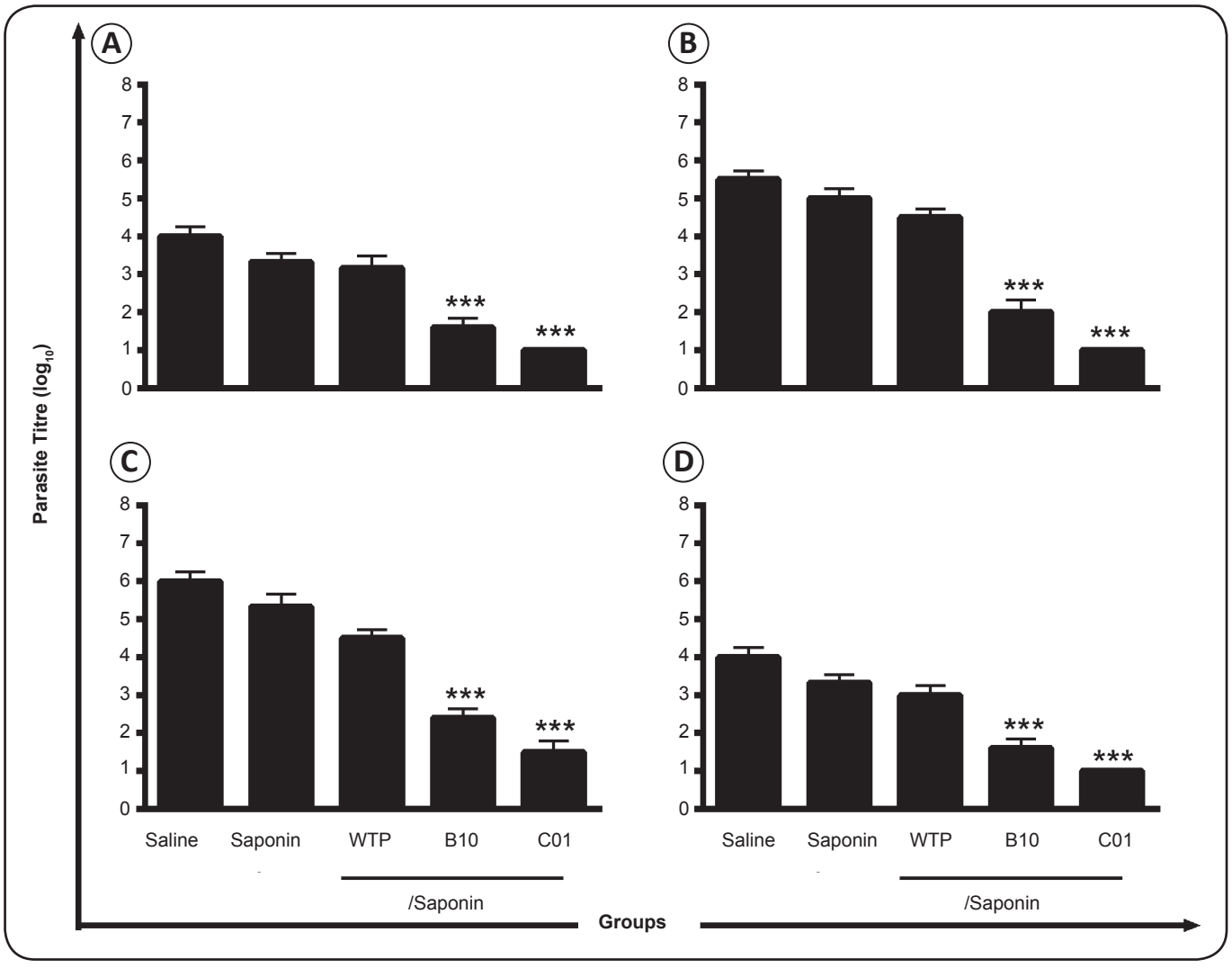

FIGURE 4 - Vaccination with candidate phage clones protects BALB/c mice against *Leishmania infantum* infection. BALB/c mice were inoculated with saline or immunized with saponin (25µg, *Quillaja saponaria* bark saponin; Sigma-Aldrich) or with the bacteriophages (1×10^{11} phage particles) plus saponin. 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.

hamsters most closely resembles natural infection in humans, this infection model requires specific laboratory conditions and trained personnel, which are not widely available, thus precluding general application of hamsters for initial testing of candidate vaccines against VL[80]. In contrast, BALB/c mice infected with *Leishmania donovani* or *L. infantum* is one the most widely studied VL models[81], which have been employed to characterize immune mechanisms underlying the development of organ-specific responses against different *Leishmania* species[82]. Given that the evaluation of parasite burden in several organs is recognized as an important indicator of vaccine efficacy against VL, the BALB/c model is considered suitable for the screening of *Leishmania* vaccine candidates. Nevertheless, additional investigations employing other animal models and experimental strategies are certainly necessary for the validation of bacteriophage-fused peptides as vaccine candidates prior to clinical studies.

CONCLUSIONS

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.

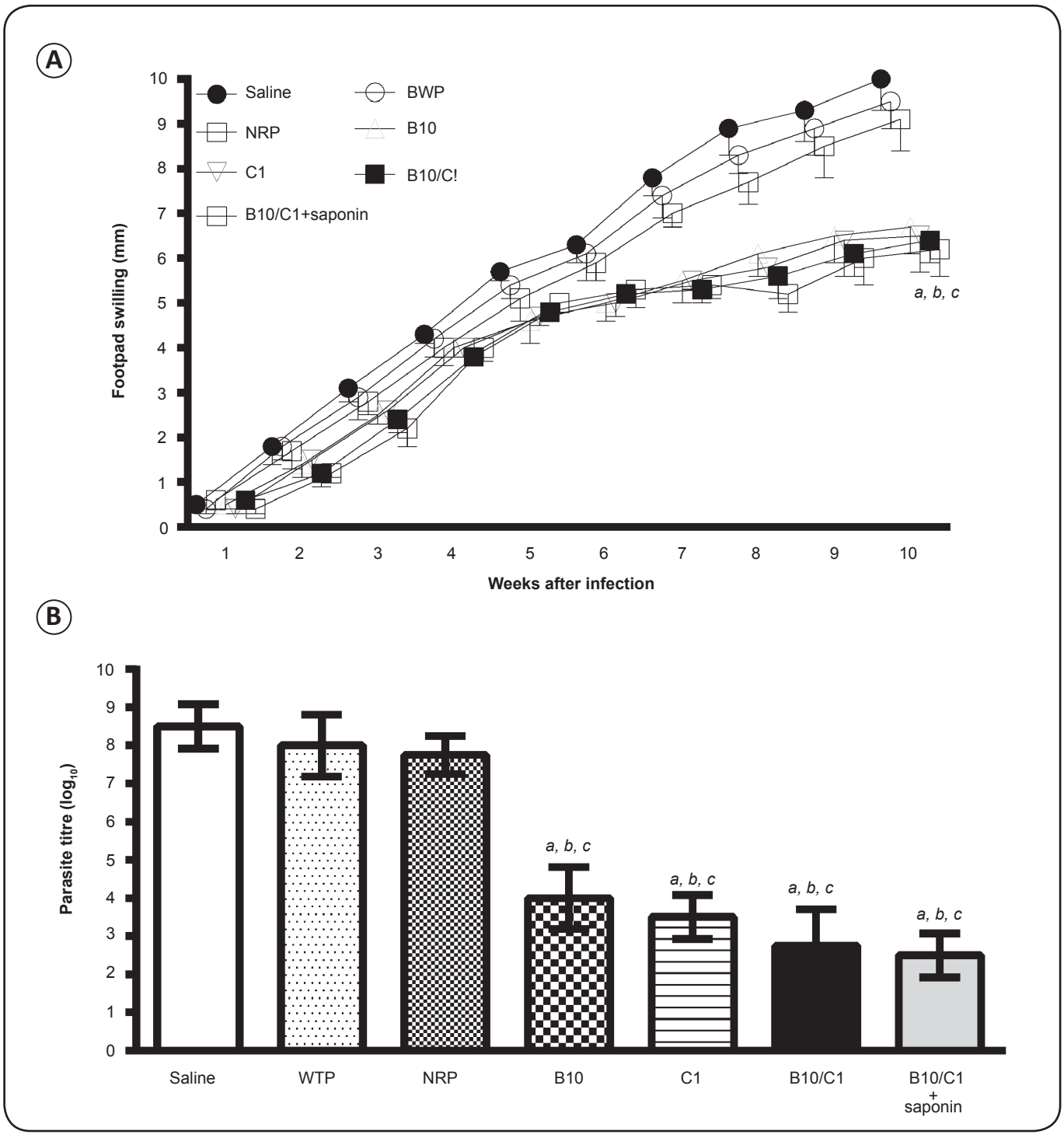

FIGURE 5 - Vaccination with candidate phage clones protects BALB/c mice against *Leishmania amazonensis* infection. BALB/c mice were inoculated with saline or immunized with bacteriophages (1×10^{11} phage particles): WTP, wild-type phage; NRP, non-relevant phage; B10 and C01, selected parasite-specific 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.

REFERENCES

1. Ashford RW. The leishmaniases as emerging and reemerging zoonoses. Int J Parasitol 2000; 30:1269-1281.
2. Gramiccia M, Gradoni L. The current status of zoonotic leishmaniases and approaches to disease control. Int J Parasitol 2005; 35:1169-1180.
3. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol 2008; 24:324-330.
4. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PloS One 2012; 7:1-12.
5. Cruz I, Acosta L, Gutiérrez MN, Nieto J, Cañavate C, Deschutter J, et al. A canine leishmaniasis pilot survey in an emerging focus of visceral leishmaniasis: Posadas (Misiones, Argentina). BMC Infect Dis 2010; 10:342-345.
6. Duprey ZH, Steurer FJ, Rooney JA, Kirchhoff LV, Jackson JE, Rowton ED, et al. Canine visceral leishmaniasis, 2000-2003. Emerg Infect Dis 2006; 12:440-446.
7. Thakur CP, Sinha GP, Pandey AK, Kumar N, Kumar P, Hassan SM, et al. Do the diminishing efficacy and increasing toxicity of sodium stibogluconate in the treatment of visceral leishmaniasis in Bihar, India, justify its continued use as a first-line drug? An observational study of 80 cases. Ann Trop Med Parasitol 1998; 92:561-569.
8. Sundar S, More DK, Singh MK, Singh VP, Sharma S, Makharia A, et al. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin Infect Dis 2000; 31:1104-1107.
9. Croft SL, Coombs GH. Leishmaniasis-current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol 2003; 19:502-508.
10. World Health Organization (WHO). Control of the leishmaniases: report of a meeting of the 399 WHO Expert Committee on the Control of Leishmaniases. (Cited 2015 June). Available at: http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf. 2010
11. Egger SS, Meier S, Leu C, Christen S, Gratwohl A, Krähenbühl S, et al. Drug interactions and adverse events associated with antimycotic drugs used for invasive aspergillosis in hematopoietic SCT. Bone Marrow Transplant 2009; 45:1197-1203.
12. Tonelli RR, Colli W, Alves MJM. Selection of binding targets in parasites using phage-display and aptamer libraries in vivo and *in vitro*. Front Immunol 2012; 3:1-16.
13. Clark JR, March JB. Bacteriophage-mediated nucleic acid immunisation. FEMS Immunol Med Microbiol 2004; 40:21-26.
14. Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 1985; 228:1315-1317.
15. Rodi DJ, Makowski L. Phage display technology-finding a needle in a vast molecular haystack. Curr Opin Biotechnol 1999; 10:87-93.
16. Sidhu SS. Phage display in pharmaceutical biotechnology. Curr Opin Biotechnol 2000; 11:610-616.
17. Manoutcharian K, Gevorkian G, Cano A, Almagro JC. Phage displayed biomolecules as preventive and therapeutic agents. Curr Pharm Biotechnol 2001; 2:217-223.
18. Matthews LJ, Davis R, Smith GP. Immunogenicity fit subunit vaccine components via epitope discovery from natural peptide libraries. J Immunol 2002; 169:837-846.
19. Hiemstra HS, Van Veelen PA, Schloot NC, Geluk A, Van Meijgaarden KE, Willemen SJ, et al. Definition of natural T cell antigens with mimicry epitopes obtained from dedicated synthetic peptide libraries. J Immunol 1998; 161:4078-4082.
20. Crameri R, Suter M. Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production. Gene 1993; 137:69-75.
21. Bakhshinejad B, Sadeghizadeh M. Bacteriophages and their applications in the diagnosis and treatment of hepatitis B virus infection. World J Gastroenterol 2014; 20:11671-11683.
22. Greenwood J, Willis A, Perham R. Multiple display of foreign peptides on a filamentous bacteriophage. Peptides from *Plasmodium falciparum* circum-sporozoite protein as antigens. J Mol Biol 1991; 220:821-827.
23. Monette M, Opella S, Greenwood J, Willis A, Perham R. Structure of a malaria parasite antigenic determinant displayed on filamentous bacteriophage determined by NMR spectroscopy: implications for the structure of continuous peptide epitopes of proteins. Protein Sci 2001; 10:1150-1159.
24. Beghetto E, Spadoni A, Buffolano W, Pezzo M, Minenkova O, Pavoni E, et al. Molecular dissection of the human B-cell response against *Toxoplasma gondii* infection by lambda display of cDNA libraries. Int J Parasitol 2003; 33:163-173.
25. Cunha-Junior JP, Silva DA, Silva NM, Souza MA, Souza GR, Prudencio CR, et al. A4D12 monoclonal antibody recognizes a new linear epitope from SAG2A *Toxoplasma gondii* tachyzoites, identified by phage display bioselection. Immunobiology 2010; 215:26-37.
26. Larralde OG, Martinez R, Camacho F, Amin N, Aguilar A, Talavera A, et al. Identification of hepatitis A virus mimotopes by phage display, antigenicity and immunogenicity. J Virol Methods 2007; 140:49-58.
27. Manhani MN, Ribeiro VS, Cardoso R, Ueira-Vieira C, Goulart LR, Costa-Cruz JM. Specific phage-displayed peptides discriminate different forms of neurocysticercosis by antibody detection in the serum samples. Parasite Immunol 2011; 33:322-329.
28. Feliciano ND, Ribeiro VS, Santos FA, Fujimura PT, Gonzaga HT, Goulart LR, et al. Bacteriophage-fused peptides for serodiagnosis of human strongyloidiasis. PLoS Negl Trop Dis 2014; 8:e2792.
29. Reis CF, Carneiro AP, Vieira CU, Fujimura PT, Morari EC, Silva SJ, et al. An antibody-like peptide that recognizes malignancy among thyroid nodules. Cancer Lett 2013; 335:306-313.
30. Pitcovsky TA, Mucci J, Alvarez P, Leguizamon MS, Burrone O, Alzari PM, et al. Epitope mapping of trans-sialidase from *Trypanosoma cruzi* reveals the presence of several cross-reactive determinants. Infect Immunol 2001; 69:1869-1875.
31. Santos PS, Nascimento R, Rodrigues LP, Santos FA, Faria PC, Martins JR, et al. Functional epitope core motif of the *Anaplasma marginale major* surface protein 1a and its incorporation onto bioelectrodes for antibody detection. PLoS One 2012; 7:e33045.
32. Manoutcharian K, Díaz-Orea A, Gevorkian G, Fragoso G, Acero G, González E, et al. Recombinant bacteriophage-based multiepitope vaccine against *Taenia solium* pig cysticercosis. Vet Immunol Immunopathol 2004; 99:11-24.
33. Grabowska AM, Jennings IR, Laing P, Darsley M, Jameson CL, Swift L, et al. Immunisation with phage displaying peptides representing single epitopes of the glycoprotein g can give rise to partial protective immunity to HSV-2. Virology 2000; 269:47-53.

34. Hardy B, Raiter A. A mimotope peptide-based anti-cancer vaccine selected by BAT monoclonal antibody. Vaccine 2005; 23:4283-4291.
35. González E, Robles Y, Govezensky T, Bobes RJ, Gevorkian G, Manoutcharian K. Isolation of neurocysticercosis-related antigens from a genomic phage display library of *Taenia solium*. J Biomol Screen 2010; 15:1268-1273.
36. Tan GH, Yusoff K, Seow HF, Tan WS. Antigenicity and immunogenicity of the immunodominant region of hepatitis b surface antigen displayed on bacteriophage T7. J Med Virology 2005; 77:475-480.
37. Gu Y, Li J, Zhu X, Yang J, Li Q, Liu Z, et al. *Trichinella spiralis*: characterization of phage-displayed specific epitopes and their protective immunity in BALB/c mice. Exp Parasitol 2008; 118:66-74.
38. Frenkel D, Katz O, Solomon B. Immunization against Alzheimer's B-amyloid plaques via EFRH phage administration. Proc Natl Acad Sci USA 2000; 97:11455-11459.
39. Santos PS, Sena AA, Nascimento R, Araújo TG, Mendes MM, Martins JR, et al. Epitope-based vaccines with the *Anaplasma marginale* MSP1a functional motif induce a balanced humoral and cellular immune response in mice. PLoS One 2013; 8:e60311.
40. Landon LA, Deutscher SL. Combinatorial discovery of tumor targeting peptides using phage display. J Cell Biochem 2003; 90:509-517.
41. Deutscher SL. Phage display in molecular imaging and diagnosis of cancer. Chem Rev 2010; 110:3196-3211.
42. Silva-Ribeiro V, Manhani MN, Cardoso R, Vieira CU, Goulart LR, Costa-Cruz JM. Selection of high affinity peptide ligands for detection of circulating antibodies in neurocysticercosis. Immunol Lett 2010; 129:94-99.
43. Suo S, Wang X, Zarlenga D, Bu RE, Ren Y, Ren X. Phage display for identifying peptides that bind the spike protein of transmissible gastroenteritis virus and possess diagnostic potential. Virus Genes 2015; May 27 (Epub ahead of print).
44. Alban SM, Moura JF, Thomaz-Soccol V, Bührer-Sékula S, Alvarenga LM, Mira MT, et al. Phage display and synthetic peptides as promising biotechnological tools for the serological diagnosis of leprosy. PLoS One 2014; 9:e106222.
45. Trigo G, Martins TG, Fraga AG, Longatto-Filho A, Castro AG, Azeredo J, et al. Phage therapy is effective against infection by *Mycobacterium ulcerans* in a murine footpad model. PLoS Negl Trop Dis 2013; 7:e2183.
46. Manoutcharian K, Terrazas LI, Gevorkian G, Acero G, Petrossian P, Rodrigues M, et al. Phage-displayed T-cell epitope grafted into immunoglobulin heavy-chain complementarity-determining regions: an effective vaccine design tested in murine cysticercosis. Infect Immun 1999; 67:4764-4770.
47. Gaubin M, Fanutti C, Mishal Z, Durrbach A, de Berardinis P, Sartorius R, et al. Processing of filamentous bacteriophage virions in antigen-presenting cells targets both HLA class I and class II peptide loading compartments. DNA Cell Biol 2003; 22:11-18.
48. Adhya S, Merril CR, Biswas B. Therapeutic and prophylactic applications of bacteriophage components in modern medicine. Cold Spring Harb Perspect Med 2014; 4:a012518.
49. Mason KA, Ariga H, Neal R, Valdecanas D, Hunter N, Krieg AM, et al. Targeting toll-like receptor 9 with CPG oligodeoxynucleotides enhances tumor response to fractionated radiotherapy targeting toll-like receptor 9 with CPG oligodeoxynucleotides enhances tumor response to fractionated radiotherapy. Clin Cancer Res 2005; 11:361-369.
50. Hashiguchi S, Yamaguchi Y, Takeuchi O, Akira S, Sugimura K. Immunological basis of M13 phage vaccine: regulation under MyD88 and TLR9 signaling. Biochem Biophys Res Commun 2010; 402:19-22.
51. Gao W, He W, Zhao K, Lu H, Ren W, Du C, et al. Identification of NCAM that interacts with the PHE-CoV spike protein. Virol J 2010; 7:254.
52. Zampoli M, Giantomassi J, Baldini S, Langoni H. *Leishmania spp.* and/or *Trypanosoma cruzi* diagnosis in dogs from endemic and nonendemic areas for canine visceral leishmaniasis. Vet Parasitol 2009; 164:118-123.
53. Silva DA, Madeira MF, Teixeira AC, Souza CM, Figueiredo FB. Laboratory tests performed on *Leishmania* seroreactive dogs euthanized by the leishmaniasis control program. Vet Parasitol 2011; 179:257-261.
54. Viol MA, Lima VMF, Aquino MCC, Gallo IG, Gallo IP, Alves D, et al. Detection of cross infections by *Leishmania spp* and *Trypanosoma spp.* in dogs using indirect immunoenzyme assay, indirect fluorescent antibody test and polymerase chain reaction. Parasitol Res 2012; 111:1607-1613.
55. Machado RZ. A study of cross-reactivity in serum samples from dogs positive for *Leishmania spp.*, *Babesia canis* and *Ehrlichia canis* in enzyme-linked immunosorbent assay and indirect fluorescent antibody test. Rev Bras Parasitol 2008; 11:7-11.
56. Strauss-Ayali D, Jaffe CL, Burshtain O, Gonen L, Baneth G. Polymerase chain reaction using noninvasively obtained samples, for the detection of *Leishmania infantum* DNA in dogs. J Infect Dis 2004; 189:1729-1733.
57. Mettler M, Grimm F, Capelli G, Camp H, Deplazes D. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. J Clin Microbiol 2005; 43:5515-5519.
58. Courtenay O, Quinnell RJ, Garcez LM, Shaw JJ, Dye C. Infectiousness in a cohort of brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. J Infect Dis 2002; 186:1314-1320.
59. Reis AB, Teixeira-Carvalho A, Vale AM, Marques MJ, Giunchetti RC, Mayrink W, et al. Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by *Leishmania (Leishmania) chagasi*. Vet Immunol Immunopathol 2006; 112:102-116.
60. Coelho EAF, Ramírez L, Costa MAF, Coelho VTS, Martins VT, Chávez-Fumagalli MA, et al. Specific serodiagnosis of canine visceral leishmaniasis using *Leishmania* species ribosomal protein extracts. Clin Vaccine Immunol 2009; 16:1774-1780.
61. Costa LE, Lima MIS, Chávez-Fumagalli MA, Menezes-Souza D, Martins VT, Duarte MC, et al. Subtractive phage display selection from canine visceral leishmaniasis identifies novel epitopes that mimic *Leishmania infantum* antigens with potential serodiagnosis applications. Clin Vaccine Immunol 2014; 21:96-106.
62. Fernandes AP, Costa MMS, Coelho EAF, Michalick MSM, Freitas E, Melo, MN, et al. Protective immunity against challenge with *Leishmania (Leishmania) chagasi* in beagle dogs vaccinated with recombinant A2 protein. Vaccine 2008; 26:5888-5895.
63. Chávez-Fumagalli MA, Martins VT, Testasicca MC, Lage DP, Costa LE, Lage PS, et al. Sensitive and specific serodiagnosis of *Leishmania infantum* infection in dogs by using peptides selected from hypothetical proteins identified by an immunoproteomic approach. Clin Vaccine Immunol 2013; 20:835-841.
64. Modabber F. Leishmaniasis vaccines: past, present and future. Int J Antimicrob Agents 2010; 36S:58-61.
65. Das A, Ali N. Vaccine prospects of killed but metabolically active *Leishmania* against visceral leishmaniasis. Expert Rev Vaccines 2012; 11:783-785.

66. Ramírez L, Santos DM, Souza AP, Coelho EA, Barral A, Alonso, C, et al. Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis. Vaccine 2013; 31:1312-1319.

67. Costa LE, Goulart LR, Pereira NCJ, Ingrid M, Lima S, Duarte MC, et al. Mimotope-based vaccines of *Leishmania infantum* antigens and their protective efficacy against visceral leishmaniasis. Plos One 2014; 9:e110014.

68. Ramirez L, Villen LC, Duarte MC, Chávez-Fumagalli MA, Valadares DG, Santos DM, et al. Cross-protective effect of a combined L5 plus L3 *Leishmania major* ribosomal protein based vaccine combined with a Th1 adjuvant in murine cutaneous and visceral leishmaniasis. Parasit Vectors 2014; 2:3-10.

69. Martins VT, Chávez-Fumagalli MA, Costa LE, Canavaci AMC, Martins AMCC, Lage PS, et al. Antigenicity and protective efficacy of a *Leishmania* amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. PLoS Negl Trop Dis 2013; 7:e2148.

70. Dey R, Dagur PK, Selvapandiyan A, McCoy JP, Salotra P, Duncan R, et al. Live attenuated *Leishmania donovani* p27 gene knockout parasites are nonpathogenic and elicit long-term protective immunity in BALB/c mice. J Immunol 2013; 190:2138-2149.

71. Rosa R, Marques C, Rodrigues OR, Santos-Gomes GM. Immunization with *Leishmania infantum* released proteins confers partial protection against parasite infection with a predominant Th1 specific immune response. Vaccine 2007; 25:4525-4532.

72. Iborra S, Parody N, Abánades DR, Bonay P, Prates D, Novais FO, et al. Vaccination with the *Leishmania major* ribosomal proteins plus CpG oligodeoxynucleotides induces protection against experimental cutaneous leishmaniasis in mice. Microbes Infect 2008; 10:1133-1141.

73. Coelho EAF, Tavares CA, Carvalho FA, Chaves KF, Teixeira KN, Rodrigues RC, et al. Immune responses induced by the *Leishmania (Leishmania) donovani* A2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania (Leishmania) amazonensis* infection. Infect Immun 2003; 71:3988-3994.

74. Goto Y, Bhatia A, Raman VS, Liang H, Mohamath R, Picone AF, et al. KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. Clin Vaccine Immunol 2011; 18:1118-1124.

75. Zanin FH, Coelho EA, Tavares CA, Marques-da-Silva EA, Costa MMS, Resende SA, et al. Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against *Leishmania chagasi* and *Leishmania amazonensis* experimental infections. Microbes Infect 2007; 9:1070-1077.

76. Carrión J, Folgueira C, Alonso C. Immunization strategies against visceral leishmaniosis with the nucleosomal histones of *Leishmania infantum* encoded in DNA vaccine or pulsed in dendritic cells. Vaccine 2008; 26:2537-2544.

77. Spitzer N, Jardim A, Lippert D, Olafson RW. Long-term protection of mice against *Leishmania major* with a synthetic peptide vaccine. Vaccine 1999; 17:1298-1300.

78. Basu R, Roy S, Walden P. HLA class I-restricted T cell epitopes of the kinetoplastid membrane protein-11 presented by *Leishmania donovani*-infected human macrophages. J Infect Dis 2007; 195:1373-1380.

79. Barral A, Pedral-Sampaio D, Grimaldi Jr G, Momen H, McMahon-Pratt D, Ribeiro-de-Jesus A, et al. Leishmaniasis in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease. Am J Trop Med Hyg 1991; 44:536-546.

80. Gomes R, Teixeira C, Teixeira MJ, Oliveira F, Menezes MJ, Silva C, et al. Immunity to a salivary protein of a sand fly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. Proc Natl Acad Sci USA 2008; 105:7845-7850.

81. Carrión J, Nieto A, Iborra S, Iniesta V, Soto M, Folgueira C, et al. Immunohistological features of visceral leishmaniasis in BALB/c mice. Parasite Immunol 2006; 28:173-183.

82. Oliveira DM, Valadares DG, Duarte MC, Costa LE, Martins VT, Gomes RF, et al. Evaluation of parasitological and immunological parameters of *Leishmania chagasi* infection in BALB/c mice using different doses and routes of inoculation of parasites. Parasitol Res 2012; 110:1277-1285.