**The antibiotic resistance-free mammalian expression plasmid vector
pPAL for development of third generation vaccines.**

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ABSTRACT

DNA vaccines require a vector to replicate genes and express encoding antigens. Antibiotic resistance genes are often used as selection markers, which must not be released to the environment upon final product commercialization. For this reason, generation of antibiotic resistance-free vectors is imperative. The pPAL vector contains the cytomegalovirus enhancer and promoter for expression in mammalian cells, and the *E. coli fabI* chromosomal gene as a selectable marker. The *fabI* gene encodes the enoyl-ACP reductase (FabI). The bacteriostatic compound triclosan is an inhibitor of this enzyme. Therefore, selection of positive clones depends of the enzyme:inhibitor molar ratio. According to western blot analysis, the pPAL vector is functional for mammalian expression of the *Leishmania infantum* (Kinetoplastid: Trypanosomatidae) gene encoding the protein kinase C receptor analogue (LACK/p36) in the HEK293T cell line transfected with pPAL-LACK. The *fabI* gene sequence contains a 210 bp CpG island, suggesting a potential role as an adjuvant of the antibiotic resistance-free pPAL vector. In fact, Th1 response induction levels against canine leishmaniasis only using pPAL-LACK was shown to be as strong as in previous strategies using a recombinant vaccinia virus in combination with standard mammalian expression plasmid vectors. In summary, the pPAL plasmid contains the essential elements for manipulation and expression of any cloned DNA sequence in prokaryotic and mammalian cells using an *E. coli* endogenous gene as a selectable marker which also provides a broad CpG island. This element enhances Th1 immune response against *L. infantum* infection in dogs using the gene encoding for the LACK antigen. Therefore, this antibiotic resistance-free plasmid is a vaccine vector actively participating in protection against canine leishmaniasis, and may be potentially tested as a vaccine vector with other antigens against different pathogens.

1. Introduction

Third generation vaccines consist of replicative DNA systems based on plasmid, cosmid or viral vectors which contain one or more genes that encode for antigens and include elements allowing expression in mammalian cells (Khan, 2013; Koprowski and Weiner, 1998). The advantages of DNA vaccines are, among others: i) long term persistence of the immunogen (Bigot and Charbit, 2009), which is convenient for chronic diseases; ii) induction of cellular responses (Khan, 2013; Koprowski and Weiner, 1998; Redding and Weiner, 2009) via MHC-I and MHC-II (Dobano et al., 2007); iii) possibility of Th1/Th2 polarization (Ferreira et al., 2008; Martinez et al., 1997; Sin et al., 1999; Weeratna et al., 2001); iv) cost-effectiveness; and v) avoidance of cold chain maintenance (Gurunathan et al., 2000; Hasson et al., 2015). However, these vaccines may contain harmful elements for the environment and/or any living organism. This is the case of antibiotic resistance genes, which must not be distributed for any purpose other than research nor released to the environment.

During the last two decades, we have developed a DNA vaccine based on the gene encoding the 36 kDa activated protein kinase C receptor analogue (LACK/p36) cloned in the pCI-neo (Promega) mammalian expression plasmid vector and recombinant vaccinia virus (rVV) or the modified rVV strain Ankara (MVA) (Ramiro et al., 2003; Ramos et al., 2008; Ramos et al., 2009). When administered in a heterologous prime-boost regime using pCI-neo-LACK and rVV-LACK or MVA-LACK, respectively, the vaccine confers partial protection in Beagle dogs against infection by *Leishmania infantum* (Kinetoplastida: Trypanosomatidae), measured as reduction (~60%) of the parasite burden in bone marrow (Ramiro et al., 2003; Ramos et al., 2008; Ramos et al., 2009). *L. infantum* is the etiological agent of zoonotic visceral leishmaniasis in the Mediterranean basin and South America (WHO, 2010). This

76 pathogen caused an outbreak in central Spain during the last lustrum (Gonzalez et al.,
77 2017; Martin-Martin et al., 2014; Molina et al., 2012). Promastigotes develop in the gut
78 of sand fly vectors (Diptera: Psychodidae), whereas amastigotes grow within
79 parasitophorous vacuoles of mammalian phagocytes (Alexander and Vickerman, 1975;
80 Chang, 1981). Due to success of proof-of-concept experiments (Ramiro et al., 2003;
81 Ramos et al., 2008; Ramos et al., 2009), the antibiotic-resistance genes *npt* and *bla*,
82 which respectively encode the neomycin phosphotransferase and the penicillinase or β -
83 lactamase, were replaced by the *E. coli* enoyl-ACP reductase gene (*fabI*) as explained
84 herein. The enoyl-ACP reductase (FabI) participates in fatty acid biosynthesis
85 catalyzing elongation (Bergler et al., 1994). The protein product is inhibited by 5-
86 chloro-2-(2,4-dichlorophenoxy) phenol, which is a bacteriostatic diphenyl ether
87 commercially called triclosan or irgasan. This compound does not have the antibiotics
88 features (Russell, 2003). The basis of this selection system is that bacterial cell growth
89 depends of the enzyme:inhibitor molar ratio. The *fabI* gene is located in the bacterial
90 chromosome and is constitutively expressed. When micromolar order concentrations of
91 triclosan are added (Goh and Good, 2008), the FabI substrate site is blocked through
92 formation of a FabI/NAD⁺/triclosan ternary complex (Heath et al., 1999). Engineering
93 the recipient bacterial strain to produce FabI enzyme excess leads to survival. For
94 example, introducing a *fabI* gene copy allows for bacterial survival to external triclosan
95 concentrations comprised between 0.5 and 5 μ M (Goh and Good, 2008). The present
96 work aims at implementing this non-antibiotic marker in a mammalian expression
97 system. We have generated the mammalian expression plasmid vector pPAL and have
98 tested the system using the protective LACK antigen-encoding gene in the canine
99 model. LACK gene expression has been verified *in vitro* using a mammalian cell line.
100 Implementing pPAL as a LACK vector achieves similar protection levels as applying

heterologous inoculation regimes using the aforementioned plasmids and viral vectors. Therefore, safety is considerably improved because antibiotic resistance markers and viral vectors are removed from the pipeline. We suggest that the pPAL vector is ready for development of DNA vaccines against any pathogen in the future. So far, this has been proven only using the LACK antigen.

2. Materials and methods

2.1. Generation of the pPAL-LACK construct

Genomic DNA was isolated from the *E. coli* strain BL21 (DE3) as explained next (Green and Sambrook, 2012). Cells were grown at 37°C in 10 mL LB medium (10 g/L triptone, 5 g/L yeast extract, 10 g/L NaCl, autoclaved at 120 °C and 1.05 Kg/cm² for 30 min) for 16 h, diluted as much as necessary in order to reach OD_{550nm} = 0.1 in a final volume of 25 mL, allowed to grow to OD_{550nm} = 0.5 (mid exponential phase), and centrifuged at 15,000g for 1 min. The sediment was washed with 50 mL of TES buffer (50 mM NaCl, 50 mM TrisHCl pH8.0, 5 mM EDTA), centrifuged again and resuspended in 100 µL of lysis buffer (25% sucrose, 40 µg/mL RNase A, 1 mg/mL lysozyme, 0.1M NaCl, 50mM TrisHCl pH8.0, 10 mM EDTA). Lysis was allowed for 20 min and then 100 µL of 2% SDS were added. The mixture was vortexed for 3 min and four freeze-thaw cycles were applied using liquid nitrogen. Subsequently, 10 µg/mL of proteinase K were added and 30 °C incubation was allowed for 30 min. Then, the sample was diluted to 500 µL with milliQ water and extractions with one volume of 25:24:1 phenol:chloroform:isoamyl alcohol and one volume of 24:1 chloroform:isoamyl alcohol were carried out. In both cases, centrifugation was performed at 15,000g for 5min. DNA was precipitated with 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of cold absolute ethanol at -20 °C for 30 min, washed with 500 µL of 70% ethanol, air dried and resuspended in 350 µL of TE buffer.

The *fabI* gene was PCR amplified including the promoter by using the oligonucleotides *PacI-fabI*-promoter-Fw and *PacI-fabI*-Rv ((**Table 1**) at 0.3 μ M each, together with 0.2 ng/ μ L *E. coli* BL21 genomic DNA, HiFi buffer, 0.3mM dNTP mix and 0.2 U/ μ L HiFi HotStart DNA polymerase (KAPA Biosystems) in a 25 μ L final reaction volume. Thermal cycling was: 95 $^{\circ}$ C, 5 min; 25 x [98 $^{\circ}$ C, 20 sec; 48 $^{\circ}$ C, 15 sec; 72 $^{\circ}$ C, 30 sec]; 72 $^{\circ}$ C 5 min. The PCR product (1131 pb) was checked by means of 1% agarose gel electrophoresis, diluted with milliQ water, purified by extraction with phenol:chloroform:isoamyl alcohol and precipitation with ethanol as detailed above, resuspended in 20 μ L of milliQ water, and quantified by UV spectrophotometry.

The purified PCR product was digested at 24 ng/ μ L at 37 $^{\circ}$ C for 2h with *PacI* restriction endonuclease in 20 μ L of reaction mixture containing NEB1 buffer, 0.1 mg/mL bovine serum albumin (BSA) and 0.4 U/ μ L *PacI* (New England BioLabs). The product was electrophoresed and gel extraction was performed with QIAquick Gel Extraction Kit (Roche) following the manufacturer's instructions. The purified product was dried in a vacuum centrifuge, resuspended in 11 μ L milliQ water, dialyzed over an MF-Millipore DNA Filter Paper membrane disc of 2.5 cm in diameter and quantified by UV spectrophotometry and densitometry after 1% agarose gel electrophoresis.

A pCI-neo-LACK plasmid (Ramiro et al., 2003) fragment was PCR amplified keeping the *bla* gene but excluding the *npt* gene by using the primers *PacI*-pCIbla-1 and *PacI*-pCIbla-2 (**Table 1**). The reaction was performed as specified above, except for primer concentration (40 pg/ μ L), hybridization temperature (60 $^{\circ}$ C) and extension time (2 min). The PCR product (pCI-bla-LACK) was purified by phenolic extraction and digested with *PacI* as detailed above, except for the final volume (100 μ L) and enzyme concentration (0.8 U/ μ L). The amplified and digested pCI-neo-LACK plasmid fragment

(5701 bp), now lacking the *npt* gene, was purified and quantified following the same procedure as for the insert.

The ligation reaction was performed at 16 °C at a 5:1 insert:vector molar ratio for 16 h in a final volume of 15 µL containing 13.3 ng/µL *fabI* insert, 75 ng pCI-bla-LACK vector, T4 DNA ligase buffer and 26.7 Weiss U/µL T4 DNA ligase (New England BioLabs). DNA was precipitated as described above and resuspended in 2 µL milliQ water. A 20 µL aliquot of electrocompetent DH5α *E. coli* cells¹ was transformed with the ligation product by electroporation. First, DNA and cells were mixed and cooled at 4 °C, as well as 1 mm electroporation cuvettes (*Cell Projects*). Then, a 1.8 kV electric pulse was applied for 5.4-5.8 ms in a *Micropulser*TM (Bio-Rad) electroporator. The default capacitance provided by the electroporation apparatus is 10 µF. Immediately, 1 mL of SOC medium (Life Technologies) was added to the mixture, which was then incubated at 37 °C for 1 h with orbital stirring at 220 rpm. A 10% aliquot was plated onto LB-agar containing 100 µg/mL ampicillin, and incubated at 37 °C for 16 h. Clone propagation was performed in liquid LB containing the antibiotic. Plasmid DNA was purified using High Pure Plasmid Isolation Kit (Roche), following the instructions provided by the manufacturer. All primers detailed above were used to sequence the corresponding regions of the pPAL-bla-LACK construct by the Sanger method.

In order to remove the *bla* gene from pCI-bla-LACK, the construct was PCR amplified with primers *Bam*HI-pCI-Δbla-1 and *Bam*HI-pCI-Δbla-2 (**Table 1**) as specified above, except for hybridization temperature (58 °C) and extension time (2 min 30 sec). The product (4805 bp) was then isolated by phenolic extraction, digested with

¹ Preparation of electrocompetent cells: a 20 mL overnight culture was diluted to 600 mL and grown to OD_{550nm} = 0.5, cooled on ice for 30 min and centrifuged at 4.000g for 10 min at 4 °C; the cell pellet was washed twice in 25 mL 1 mM HEPES pH7.0, resuspended in 24 mL of 10% glycerol and distributed in 20 µL aliquots, which were stored at -80 °C.

*Bam*HI, purified, quantified and re-ligated following the procedures detailed previously. Re-ligation was performed at a construct concentration of 32 ng/μL in a final volume of 10 μL. Chemically competent *E. coli* cells of the SURE strain (Agilent Technologies) were transformed by heat shock following the manufacturer's instructions. This strains' genotype (*McrA*⁻, *McrCB*⁻, *McrF*⁻, *Mrr*⁻, *HsdR*⁻, *endA*⁻, *recB*⁻, *recJ*) avoids unwanted rearrangements. Selection was performed with 3 μM triclosan (Sigma). Finally, the whole construction was sequenced with all six primers detailed above plus the following (**Table 1**): pCIseq1; 106-LACK-pCIseq; LACK-Fw; LACK-Rv; pCIseq2; pCIseq3; *Xba*I-pCIseq4.

2.2. Generation of the pPAL plasmid vector

The LACK gene had been originally cloned into the pCI-neo vector *Eco*RI and *Xba*I targets (Ramiro et al., 2003). This procedure eliminated the *Mlu*I restriction site of the polylinker, which is obviously absent in the pPAL-LACK construct. In order to obtain the pPAL plasmid vector, the *Mlu*I site was included and the LACK gene excluded by using primers *Xba*I-pCIseq4 and *Xba*I-*Mlu*I-*Eco*RI-pCI-Fw (**Table 1**) for PCR amplification. Purification, *Xba*I digestion, re-ligation, transformation, clone selection, and sequencing procedures were performed as described above.

CpG islands were predicted in the entire sequence of pPAL with CpG Island Searcher Software (Takai and Jones, 2002, 2003) using default parameters.

2.3. HEK 293T cell line transfection

The HEK 293T cell line (ATCC CRL-3216) was cultured until reaching semi-confluence (~48 hrs) at 37 °C in the presence of 5% CO₂ in pre-warmed complete medium (CM) containing DMEM (Gibco BRL) medium supplemented with 10% heat-inactivated fetal bovine serum (Lonza) and penicillin 100 IU/mL-streptomycin 100 μg/mL, this being the complete medium (CM). After propagation, cells were detached

by mild washing with fresh medium, centrifuged at 250g for 10 min, resuspended in CM, distributed at a cell density of $\sim 2 \times 10^5$ cells/cm² in 1 mL aliquots in a 12-well plate, and incubated as detailed previously. Once the cell culture was semi-confluent, each plasmid was prepared for transfection as follows. First, 4 μ L of JetPei DNA transfection reagent (PolyPlus Transfection) were diluted with 46 μ L of a sterile 150 mM NaCl solution. Then, this suspension was added to a solution containing 2 μ g plasmid (pCI-neo-LACK, pORT-LACK, pPAL-LACK) dissolved in sterile 150 mM NaCl, and the same diluent without DNA was used for the mock control. After washing semi-confluent cells with DMEM, each DNA-JetPei mixture was carefully added to the cells and incubated for 5 min at room temperature. Finally, 1 mL of CM was added per well and the cells were incubated as described until cultures were confluent.

2.4. Evaluation of the LACK expression levels by Western blot

Cells were lysed with a buffer containing 25mM Tris-HCl pH 7.8, 2mM EDTA, 2mM DTT, 1% glycerol, and 1% Triton X-100 at room temperature for 5 min. Protein extracts were quantified by the Bradford method and separated by SDS-PAGE in 10% polyacrylamide gels at 12 mA for 30 min, and then at 30 mA for 2h. Transfer to a HybondTM-C pure membrane (Amersham) was carried out at 100 V for 1 h. All these procedures were performed as described (Alcolea et al., 2014). Thereafter, the membrane was blocked with 5% skimmed milk in PBS-0.05% Tween 20 (PBS-Tween) at room temperature under mild shaking for 1 h. Next, the membrane was rinsed with PBS-Tween and washed twice with the same solution for 15 and 5 min, respectively. The membrane was incubated at room temperature under mild shaking for 1 h with 1:1000-diluted rabbit anti-LACK polyclonal antibody (Gonzalez-Aseguinolaza et al., 1999) in PBS-Tween. After washing, the membrane was incubated with the HRP-conjugated goat anti-rabbit whole IgG polyclonal antibody (DAKO) at a 1:2000 dilution

in PBS-Tween. The membrane was washed, incubated with ECL Western Blotting Reagent (Amersham) for 30 s and developed on an X-ray film.

2.5. Canine leishmaniasis vaccination data source

Consent (06/04/2014, reference PI28/14) for pPAL-LACK proof-of-concept procedures was approved by the University of Zaragoza Ethical Advisory Commission for Animal Experimentation. *L. infantum* amastigote burden in bone marrow biopsy, humoral and cellular immune response in peripheral blood and target organs, and canine leishmaniasis clinical signs were evaluated (Alcolea et al., 2016).

2.6. qRT-PCR analysis of *Th1* cytokine gene expression in lymph node

Dog popliteal lymph node samples (~20 µL) were obtained in aseptic conditions by aspiration using sterile 21-gauge needles and 5 mL syringes. These samples were immediately lysed in 1 mL TRIzol reagent (Life Technologies) and incubated for 5 min at room temperature. At this point, samples could be stored at -80 °C until further processing after 1 day. The samples were allowed to thaw at room temperature, emulsified after adding 200 µL chloroform, left to stand for 2 min, and centrifuged at 15,000g at 20 °C for 15 min. The aqueous phase was carefully recovered avoiding contamination with DNA from the interface. RNA was precipitated with 0,5 mL of isopropanol at room temperature for 10 min and stored at -80 °C until further processing. Precipitated RNA was centrifuged, washed with 75% ethanol in DEPC-treated (RNase-free) water, air-dried, resuspended in RNase-free water, and quantified by UV absorption spectroscopy. RNA quality was assessed with Agilent 2100 Bioanalyzer using an RNA 6000 Nano Chip, according to the manufacturer's instructions. Summarizing, 550 µL of RNA 6000 Nano Gel Matrix was applied on top of a filter cartridge provided by the manufacturer and centrifuged at 1,500g for 10 min. RNA 6000 Nano Dye Concentrate was equilibrated at room temperature for 30 min,

vigorously mixed for 10 s, and mixed with filtered gel matrix (1:65 μ L). An RNA 6000 Nano Chip was loaded with the gel-dye mix. The samples and the molecular weight ladder were applied to the chip, and 1 μ L RNA 6000 Nano Marker was added to every well. The chip was thoroughly mixed during 1 min using an IKA vortexer and run in the bioanalyzer using Agilent 2100 Bioanalyzer Expert Software. Single-stranded cDNA was synthesized from 100 ng - 1 μ g of total RNA. First, RNA was mixed with 6 μ g of random hexamer primers (Life Technologies), denatured at 70 °C for 10 min and immediately cooled to 4 °C. Then, samples were incubated at 46 °C for 3 h with 570 μ M each dNTP, 10 μ M DTT and 600 U SuperScript® Reverse Transcriptase (Life Technologies) in a final volume of 30 μ L. RNA was degraded with 5U RNase H (Life Technologies) at 37 °C for 20 min. The enzyme was inactivated at 75 °C for 10 min. Then, the product was purified using QiaQuick PCR Purification Kit (Qiagen) following the manufacturer's instructions.

Pre-designed dog TNF- α , IFN- γ (genes of interest), and β -actin (endogenous control) TaqMan® MGB Assays (Life Technologies) were run in a 7900HT Fast Real Time PCR system (Life Technologies) using TaqMan® Universal Master Mix 2x (Life Technologies) following the procedure specified by the manufacturer. Information about primer and probe location on NCBI gene assemblies is listed in **Table 2**. The final reaction volume was 10 μ L. cDNA template final amounts per reaction were 10, 2, and 0.4 ng and were set by serial dilution. Three technical replicates of each reaction were performed in the same plate. Thermal cycling conditions were: 95 °C for 5 min; 40 x [95 °C for 30''; 60 °C for 1 min, data acquisition]. Coefficients of variation (CV) were checked ($\leq 20\%$), PCR efficiencies were calculated by the standard curve best fit method considering all technical replicates from a reaction except for outliers in case they appeared. Efficiency-corrected raw quantities were calculated as raw efficiency to the

power of $-Ct$. Then, efficiency-corrected raw quantities obtained for the genes of interest were normalized (Q_n) dividing them by the corresponding endogenous control values. Statistical inference was performed using the Mann-Whitney U-test.

3. Results and discussion

The use of antibiotic resistance markers may promote spreading antibiotic resistance traits to the environment, which would be a major problem in industrial scale preparations (Paterson, 2006). We aimed to improve safety of the pCI-neo-LACK/MVA-LACK vaccine against canine leishmaniasis before proceeding to dosage-response evaluation and analysis of long-lasting immunity. For this purpose, antibiotic resistance gene removal is essential. The *npt* gene was successfully replaced by the *fabI* gene from the pCI-neo-LACK construct by using the *bla* selection marker in the molecular cloning process, leading to the pPAL-*bla*-LACK construct. Then, the *bla* gene was removed in a second cloning step using the *fabI* selection marker, thus obtaining the pPAL-LACK construct (4,828 bp). The LACK gene was removed in order to obtain the antibiotic-free pPAL mammalian expression plasmid vector (3,899 bp). During this process, the *MluI* restriction site was restored where the LACK gene had been cloned. Therefore, multiple cloning sites of the pPAL and pCI-neo vectors are identical. Both contain the pMB1 replication origin. The process is outlined in **Figure 1**. pPAL and pPAL-LACK plasmid maps are depicted in **Figure 2**. Agarose gel electrophoresis of pPAL and pPAL-LACK purified from clones finally selected for storage and vaccine development is shown in **Figure 3A**. **Supplementary File 1** contains both complete sequences, which have been deposited in GenBank (accession numbers MG882756 and MG882757). A pPAL and a pPAL-LACK bacterial clone were selected, completely re-sequenced using all **Table 1** primers. These clones were expanded and a master cell bank was conveniently stored. Four aliquots of each master

bank were selected for sequence stability testing. For this purpose, these aliquots were subcultured three times, completely sequenced, and an aliquot of the subculture frozen. This cycle has been repeated monthly during the last six years. No rearrangements in the plasmid sequence have been observed. The pPAL and pPAL-LACK plasmid preparations contain monomers with open circular (OC) and super-coiled (SC, or covalently closed circle, CCC) topology (**Figure 3A**). pPAL preparations also contain concatenated units. The absence of bands corresponding to linear molecules (3,899 and 4,828 bp, respectively) in the agarose gel is indicative of high quality plasmid preparations, maintaining plasmid integrity as a circular molecule.

Western blot analysis performed with the rabbit anti-LACK polyclonal antibody revealed that HEK293T cells transfected with pPAL-LACK express the LACK gene yielding similar protein levels as pCI-neo-LACK and pORT-LACK transfectants (**Figure 3B** and **C**). In fact, the mock control shows no LACK protein expression, which is enabled in transfectants at high levels thanks to the cytomegalovirus (CMV) enhancer and promoter. Western blotting using the anti-LACK polyclonal antibody shows bands corresponding to protein molecules with a molecular weight higher than 36 kDa (**Figure 3C**). This is probably due to unspecific recognition of epitopes in human cells. However, this probable artifact does not affect specific detection of the 36-kDa LACK protein according to comparison of test samples with mock controls. In view of these results, pPAL vector is ready to generate potentially functional third generation vaccines against any pathogen, although only the LACK gene has been tested using this vector.

CpG island prediction in the sequence of the pPAL plasmid was performed with CpG Island Searcher Software (Takai and Jones, 2002, 2003). This analysis revealed that the *E. coli fabI* gene sequence including the promoter contained a single CpG island

between positions 700 and 909 containing 59% G+C
 (AAAAGCTTGCCGCTCCATGCTGAATCCGGGTCTGCCCTGCTGACCCTTCC
 TACCTTGGCGCTGAGCGCGCTATCCCGAACTACAACGTTATGGGTCTGGCAA
 AAGCGTCTCTGGAAGCGAACGTGCGCTATATGGCGAACGCGATGGGTCCGG
 AAGGTGTGCGTGTTAACGCCATCTCTGCTGGTCCGATCCGTACTCTGGCGGC
 TTC). We postulate that this CpG island is immunostimulatory and may potentially act
 as an adjuvant because CpG-rich motifs have been described to enhance Th1 responses
 driven by IFN- γ and IL-12 (Cowdery et al., 1996; Halpern et al., 1996), which is
 desirable in protection against intracellular pathogens such as *Leishmania* spp. In fact,
 vaccination studies have shown *L. infantum* amastigote burden reduction in bone
 marrow of Beagle dogs after immunization using a pPAL-LACK homologous regime
 and a 10^8 *L. infantum* promastigote infectious challenge is accompanied by increase of
 the Th1 cytokines IFN- γ and TNF- α (**Figure 4**), as shown in WO2016188973A1
 European Patent report (Alcolea et al., 2016). The box plots depicted in **Figure 4** show
 that both cytokines are increased 300 days post-infection (dpi) in pPAL-LACK
 vaccinated dogs with respect to control dogs. Test group medians approximately double
 control group medians, and upper limits are much higher in the test control, showing
 more outliers of higher Qn values (7 to 50 times higher) and higher third quartiles
 (**Figure 4**). The non-parametric Mann-Whitney U-test was used because populations are
 heterogenous and groups small (10 dogs per group). These conditions are inherent to
 experimentation with the canine model as a consequence of genetic variability and
 because specialized and expensive lodging facilities and trained personnel are required.
 The results obtained indicate that IFN- γ and TNF- α increments induced in the pPAL-
 LACK vaccinated dog group with respect to the control group are statistically
 significant ($p = 0.0316$ and $p = 0.0321$, respectively).

Parasite burden decrease (~60%) and Th1 cytokine increase is similar using the pCI-neo-LACK/rVV-LACK or pCI-neo-LACK/MVA-LACK regime system (Alcolea et al., 2016; Ramiro et al., 2003; Ramos et al., 2008; Ramos et al., 2009). Therefore, pPAL-LACK vaccine confers about the same protection levels and adds two essential benefits for safety upon future commercialization after manufacture in GMP conditions: no release of antibiotic resistance genes and recombinant viruses to the environment. These are important advances in safety. In the pPAL-LACK example, two construct doses would be inoculated to the animals and the rVVA-LACK and MVA-LACK recombinant viruses would be removed from the pipeline. In summary, the *fabI* gene CpG island enhances the Th1 response and increases protection conferred by the plasmid vaccine. This is an important step of the last pPAL-LACK vaccine development stages, which is important for canine leishmaniasis control. In fact, dogs act as the reservoirs in zoonotic visceral leishmaniasis cycle, which is a public health problem in the mediterranean basin and South America. The *fabI* non-antibiotic resistance marker has also been tested in a vaccine developed against the bovine viral diarrhoea virus (BVDV) using the E2 antigen-encoding gene. The circulating antibody profile observed was similar when using the *fabI* and the *bla* markers (El-Attar et al., 2012). Therefore, the *fabI* marker has been demonstrated to be a good substitute of antibiotic resistance markers because it does not affect (BVDV) or even improves (*L. infantum*) the immune response against the pathogen.

4. Conclusions.

pPAL is an antibiotic-free mammalian expression plasmid vector suitable for development of third generation vaccines, including therapeutic vaccines, which is based on the CMV enhancer/promoter expression system and the *fabI*/triclosan selection system. This vector may also act as an adjuvant because the *fabI* gene contains

a CpG island covering approximately one third of the gene sequence including the promoter. The benefits of this CpG island have been proven in vaccination tests against canine leishmaniasis.

Abbreviations

ACP, acyl carrier protein; BSA, bovine serum albumin; CMV, cytomegalovirus; IFN- γ , interferon γ ; GMP, Good Manufacturing Practice; LACK/p36, analogue of the receptor of the activated protein kinase C; MVA, modified rVV strain Ankara; *npt*, neomycin phosphotransferase gene; *fabI*, enoyl-ACP-reductase gene; rVV, recombinant vaccinia virus; TNF- α , tumoral necrosis factor α .

Competing interests.

The authors declare that they do not have competing interests.

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FIGURE LEGENDS

Figure 1. Outline of the cloning process.

Figure 2. Physical maps of the pPAL mammalian expression vector and the third

generation vaccine pPAL-LACK. (A) Cloning steps for pPAL generation. **(B)** The

main elements of the pPAL plasmid are: i) the pMB1 replication origin; ii) the

cytomegalovirus (CMV) system for expression in mammalian cells, including the CMV

enhancer and promoter; iii) a chimeric intron; iv) the T7 promoter for expression in

prokaryotes; v) the polylinker or multiple cloning site (MCS); vi) the SV40 late polyA

signal activator; vii) the *E. coli fabI* gene, including the constitutive promoter. **(C)** The

pPAL-LACK recombinant plasmid is a third generation vaccine, as the LACK gene was

previously shown to protect dogs against infection by *L. infantum*. The gene was cloned

at the *EcoRI* and *XbaI* sites.

Figure 3. Heterologous expression in mammalian cells of the *L. infantum* LACK

gene cloned in the pPAL vector. (A) Electrophoresis of the pPAL and pPAL-LACK

plasmids in 1% agarose gel. The MW marker is 1Kb DNA ladder (New England

Biolabs). From lower to upper, the plasmid bands observed are: monomer in open

circular (OC) topology; monomer in super-coiled (SC, or covalently closed circle, CCC)

topology; dimers, trimers and multimers of concatenated units. The pPAL and pPAL-

LACK plasmid sizes are 3,899 bp and 4828 bp, respectively. No band is observed at

this position, meaning that linear topology is absent, or that undetectable traces of linear

plasmid may be present. Therefore, plasmid integrity as a circular molecule was

maintained. Sanger sequencing demonstrates that the sequences and sizes are as

designed (Supplementary File 1). pPAL-LACK concatenation is not observed. **(B)** and

(C) Two biological replicates of Western blot experiments showing expression

induction in the HEK293T cell line. No expression of the 36 kDa LACK protein is

observed in the mock control and similar expression levels are observed when using the pCI-neo-LACK (Ramos et al., 2008) and pORT-LACK (Ramos et al., 2009) constructions. Upper bands are probably due to unspecific recognition of epitopes in human cells, which does not affect to specific detection of the 36-kDa LACK protein according to comparison of test samples with mock controls.

Figure 4. Th1 cytokine relative levels in popliteal lymph node of control and pPAL-LACK vaccinated dogs. Cytokine levels were evaluated by real time qRT-PCR using cDNA samples synthesized from popliteal lymph node aspirate biopsies. Qn, normalized quantity values (Bookout et al., 2006). Non-parametric statistical inference was performed using the Mann-Whitney U-test ($\alpha = 0.05$). **(A)** IFN- γ Qn measured 300 days post infection (dpi); **(B)** TNF- α Qn measured 300 dpi. Figure elaborated using data from (Alcolea et al., 2016).

TABLES

Table 1. Cloning and sequencing primers. Restriction sites are underlined. Restriction sites in primer *XbaI-MluI-EcoRI*-pCI-Fw are contiguous, and *XbaI* and *MluI* sites overlap in a single-nucleotide length. These restriction sites are TCTAGA, ACGCGT, and GAATTC, respectively.

Primer name	Primer sequence	T _m (°C)
<i>PacI-fabI</i> -promoter-Fw	TACTGGATTAATTAAGTGCTGGAGAATATTCG	58
<i>PacI-fabI</i> -Rv	TACTGGATTAATTAATTATTTCAGTTCGAGTTCGTTC	59
<i>PacI</i> -pCIbla-1	TACTGGATTAATTAACCGCGTATGGTGCACCTCTCA	63
<i>PacI</i> -pCIbla-2	TACTGGATTAATTAACGCTGGCAAGTGTAGCGGT	63
<i>Bam</i> HI-pCI-Δbla-1	GTACAGGATCCCATGTGTCAGAGGTTTTTCAC	63
<i>Bam</i> HI-pCI-Δbla-2	GTACAGGATCCGCAGAAAGTGGTCCTGCAACTT	66
pCIseq1	TCAATATTGGCCATTAGCCAT	49
106-LACK-pCIseq	CCACGAGATGGCCGTGCCATC	60
LACK-Fw	ATGAACTACGAGGGTCACCT	52
LACK-Rv	TTACTCGGCGTCGGAGATG	53
pCIseq2	GTTAAGGGATTTTGGTCATGA	49
pCIseq3	TCATGACCAAAATCCCTTAAC	49
<i>XbaI</i> -pCIseq4	TCTAGAGTCGACCCGGGC	55
<i>XbaI-MluI-EcoRI</i> -pCI-Fw	TATTACTCTAGACGCGTGAATTCTCGAGGCTAGCCT	66

Table 2. Information about pre-designed TaqMan® MGB primers and probes. The primers and probe sequences are not provided by the manufacturer. The assay location is specified and defined as the nucleotide location that is the midpoint of the 25-bp context sequence for the associated accession number. The manufacturer also specifies that the 5'-FAM-3'-MGB TaqMan® NFQ probes are generally 15–18 nucleotides long and are located within the context sequence.

Target gene	Context sequence and commercial assay number	NCBI gene reference
Canine IFN-γ	CTGATTCAAATTCCTGTGAACGATC #Cf02623316_m1	NM_001003174.1,AF091130.1,AF126247.1,S41201.1,AF327901.1,EF095772.1,FJ194478.1
Canine TNF-α	CATGTTGTAGCAAACCCCGAAGCTG #Cf02624261_m1	NM_001003244.4,AF327899.1,DQ923808.1,EU249361.1
β-actin	CCAGACAGATGGAGAAGGACGAGAC #Cf02635224_m1	XM_535384.2

SUPPLEMENTARY FILES

Supplementary File 1. Plasmid sequences.

PacI-fabI-promoter-Fw

PacI-fabI-Rv

E. coli genomic DNA

PCR
Digestion
Ligation

pCI-bla-LACK

PCR
Digestion
Ligation

pPAL-LACK

XbaI-MluI-EcoRI-pCI-Fw

XbaI-pCIsq4

PCR
Digestion
Ligation

pPAL

pCI-neo-LACK

PacI-pCibla-1

PacI-pCibla-2

BamHI-pCI-Δbla-1

BamHI-pCI-Δbla-2

pMB1 ori

bla

npt

CMV e/p

LACK

fabI

A

B

A **B**

Primer name	Primer sequence	Tm (°C)
<i>PacI</i> - <i>fabI</i> -promoter-Fw	TACTGGATTAATTAAGTGCTGGAGAATATTCG	58
<i>PacI</i> - <i>fabI</i> -Rv	TACTGGATTAATTAATTATTTCAGTTCGAGTTCGTTC	59
<i>PacI</i> -pCIbla-1	TACTGGATTAATTAACCGCGTATGGTGCACCTCTCA	63
<i>PacI</i> -pCIbla-2	TACTGGATTAATTAACGCTGGCAAGTGTAGCGGT	63
<i>Bam</i> HI-pCI-Δbla-1	GTACAGGATCCCATGTGTCAGAGGTTTTAC	63
<i>Bam</i> HI-pCI-Δbla-2	GTACAGGATCCGCAGAAGTGGTCCTGCAACTT	66
pCIseq1	TCAATATTGGCCATTAGCCAT	49
106-LACK-pCIseq	CCACGAGATGGCCGTGCCATC	60
LACK-Fw	ATGAACTACGAGGGTCACCT	52
LACK-Rv	TTACTCGGCGTCGGAGATG	53
pCIseq2	GTTAAGGGATTTTGGTCATGA	49
pCIseq3	TCATGACC AAAATCCCTTAAC	49
<i>Xba</i> I-pCIseq4	TCTAGAGTCGACCCGGGC	55
<i>Xba</i> I- <i>Mlu</i> I- <i>Eco</i> RI-pCI-Fw	TATTACTCTAGACGCGTGAATTC	66