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Recombinant BCG strains expressing chimeric proteins derived from *Leptospira* protect hamsters against leptospirosis

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ABSTRACT

Leptospirosis is a zoonosis that is responsible for one million human cases per year. Fusing multiple immunogenic antigens represents a promising approach to delivering an effective vaccine against leptospirosis. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is a potential vaccine vector due to its adjuvant properties and safety. Two chimeric genes based on genic sequences of *ligANI*, *ligBrep*, *lipL32*, and *lemA*, were individually cloned into five BioBrick vectors with different promoters (pAN, Hsp60, 18 kDa, Ag85B and Ag85B plus signal sequence) for antigen expression in BCG. Groups of ten hamsters were vaccinated with recombinant BCG (rBCG) strains in two doses of 10^6 CFU and challenged with $5 \times LD_{50}$ of *L. interrogans* serovar Copenhageni. All rBCG vaccines expressing chimera 1, based on antigens LipL32, LigANI, and LemA, under the control of any promoter, protected 80–100% of the hamsters from challenge ($P < 0.05$) and four of them also protected from renal carrier status; for chimera 2, based on LigANI and LigBrep antigens, the only vaccine that afforded survival rates statistically different from the control was the vaccine that incorporated the pAN promoter (60% of survival). A single vaccine dose was sufficient to induce significant IgG levels by all vaccine compositions evaluated; however, humoral response was not related to protection. These findings suggest that the combination of potential vaccine candidates in chimeric antigens and the use of BCG as a live vector are promising strategies by which it is possible to obtain an effective and sterilizing vaccine against leptospirosis.

1. Introduction

Leptospirosis is an emerging zoonosis that is distributed throughout the world [1]. The infection, which is caused by pathogenic bacteria from the *Leptospira* genus, occurs through contact with the urine of animal reservoirs that harbor the spirochete in renal tubules [2]. Thus, the disease has been associated with occupational risks, such as veterinary medicine and farming, and with recreational activities such as water sports [3]. Moreover, environmental exposures arising from poor sanitation, poverty, and uncollected refuse, are by far the most significant risk factors in urban slum settings such as those commonly observed in developing countries like Brazil [4,5]. Clinical manifestations in humans range from a mild febrile illness to multi-organ system complications [3]. In the agricultural setting, the main manifestations of infections in large animals are reproductive problems, and these result in significant economic losses [6,7]. It is estimated to occur one million human cases of leptospirosis per year, resulting in more than 58,000 deaths [8] and 2.90 million disability-adjusted life years (DALYs) [9].

The only commercially available vaccines against leptospirosis are currently bacterins, inactivated whole-cell preparations that induce an immune response that is predominantly against lipopolysaccharide (LPS). As such, protection is limited to the serovars included in the vaccine composition and the immunity induced by bacterins is short term [10]. Multivalent vaccines are required in Brazil, where multiple serovars circulate and epidemiological surveillance is poor, especially with regards to neglected diseases [10]. Besides, bacterins are often associated with adverse reactions and, therefore, these vaccines are not particularly well suited for human use. Thus far, efforts to overcome these

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limitations have focused on the construction of recombinant vaccines based on well-conserved antigens [11].

Outer membrane proteins are considered promising vaccine targets [12,13]. The proteins LigANI, LigBRep, LipL32, and LemA have been proven to promote partial protection against homologous challenge in hamsters [11,14–16]. However, such protection does not prevent renal colonization. It may be possible to address this shortfall by fusing a combination of immunogenic antigens in a multi-epitope protein that can provide renal clearance.

Mycobacterium bovis bacillus Calmette-Guérin (BCG) is a live attenuated vaccine that is widely used to prevent tuberculosis and represents a promising alternative for antigen delivery [17]. BCG is safe, stable, and presents strong adjuvant properties. Moreover, it replicates in macrophages and dendritic cells and, therefore, can effectively present antigens to the host immune system and confer long-term immunity [18]. Several heterologous antigens, including those from *Leptospira* spp., have already been successfully expressed in recombinant BCG (rBCG) under the control of different promoters [17]. The strength of promoters may affect the levels of antigen expression and strain stability *in vivo* [19]. Recently, our group developed a mycobacterial toolbox (data not published) that contained a stock of compatible sequences constructed using the BioBricks® strategy. This method allows the construction of numerous combinations of parts using a more practical approach than that associated with conventional cloning [20].

In the current study, we report the cloning and expression of two different chimeric genes derived from *Leptospira interrogans* in BCG under the control of five different mycobacterial promoters (pAN, Hsp60, 18 kDa, Ag85B and Ag85B plus signal sequence) using previously constructed plasmids that are compatible with the BioBrick method. Moreover, we demonstrate the immunoprotective potential of these rBCG strains as live vaccines against leptospirosis in hamsters.

2. Materials and methods

2.1. Ethics statement

All animal procedures were performed at the animal facility of the Federal University of Pelotas (UFPel) and approved by the Ethics Committee for Animal Experimentation (CEEA) of UFPel, under protocol number 4646-2015. The CEEA at UFPel is accredited by the Brazilian National Council for Animal Experimentation Control (CONCEA). The animals were maintained in accordance with international guidelines throughout the experiments.

2.2. Strains and growth conditions

Escherichia coli strain DH5α was grown in Luria-Bertani medium at 37 °C with or without kanamycin to 50 $\mu g \cdot ml^{-1}$. *Mycobacterium bovis* BCG Pasteur was cultured at 37 °C in Middlebrook 7H9 medium (Difco, BD, São Paulo, SP, Brazil) with 10% of oleic acid, albumin, dextrose complex (OADC - Difco), 0.2% glycerol, and 0.05% Tween 80 or 7H10 agar (Difco, BD, São Paulo, SP, Brazil) supplemented with 10% OADC and 0.2% glycerol, and kanamycin (25 $\mu g \cdot ml^{-1}$) when necessary. *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain Fiocruz L1-130 was maintained at 30 °C in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid using *Leptospira* enrichment EMJH for supplementation (Difco, BD, São Paulo, SP, Brazil). All experiments were performed using a low-passage strain of *L. interrogans*; i.e., eight passages *in vivo* and three passages *in vitro*.

2.3. DNA manipulation reagents

Oligonucleotides were synthesized by Exxtend (São Paulo, Brazil). PCR reactions were performed using GoTaq® Colorless Master Mix (Promega, Wisconsin, United States). Restriction enzymes and T4 DNA ligase enzyme were purchased from New England Biolabs (Massachusetts, United States).

2.4. Assembly of recombinant vectors

Plasmids were previously constructed for antigen expression in BCG under control of five different promoters, named pUP500/P_{pAN}, pUP500/P_{18kDa}, pUP500/P_{hsp60}, pUP500/P_{ag85B} and pUP500/P_{sag85B} (data not published yet). The pUP500 plasmid is a shuttle vector able to replicate in *E. coli* and *M. bovis* BCG that presents resistance gene to kanamycin and multiple cloning site compatible with BioBricks standard. Briefly, the promoter sequences were amplified by PCR from genomic DNA of *M. bovis* BCG Pasteur and individually cloned into this plasmid using restriction enzymes *Eco*RI and *Pst*I. Mycobacterial promoters used in this study included pAN, 18 kDa, Hsp60 and Ag85B, that control the expression of target antigens in the cytosol, and Ag85B plus a signal sequence that drives expression for extracellular medium (abbreviated as SAg) [19]. Chimeric genes were amplified by PCR from plasmids previously constructed (manuscript submitted) using primers described in Table 1. Chimeric gene 1 (Q1) is a fusion of *lipL32* (24–272 aa), *lemA* (28–157 aa) and *ligAni* (943–1224 aa) sequences, while chimeric gene 2 (Q2) is a fusion of *ligAni* (943–1224 aa) and *ligBrep* (131–645 aa) sequences (Fig. 1). BioBrick vectors constructed by our group were digested with *Spe*I and *Pst*I and individually ligated with the chimeric genes digested with *Xba*I and *Pst*I. *E. coli* DH5α competent cells were electroporated with the ten recombinant plasmids and the cloning of chimeric sequences downstream of each promoter into pUP500 was confirmed by sequencing.

2.5. BCG transformation and analysis of expression

M. bovis BCG Pasteur electrocompetent cells were transformed with the recombinant plasmids using the method previously described by Parish & Stoker [21]. BCG transformants were

Table 1
Nucleotide sequences of primers and probes used in this work.

Primer/probe	Sequence (5′-3′)	Label	Final concentration (nM)
Q1 forward	CCGGAATTCGCGGCCGCTTCTAGAGGTGGTCTGCCAAGCCTAA	–	500
Q1 reverse	TGCACTGCAGCGGCCGCTACTAGTTTATTATGGCTCCGTTTTAATAGA	–	500
Q2 forward	CCGGAATTCGCGGCCGCTTCTAGAAGAATAGCTTCAATC	–	500
Q2 reverse	TGCACTGCAGCGGCCGCTACTAGTTTATGGAGTGAGTGTATT	–	500
lipl32 forward	TTGGATCCGTGTAGAAAGAATGTC	–	300
lipl32 reverse	TCGTCCAATTTTTGAACTGGTTT	–	300
lipl32 probe	CCAAATCGCCAAAGCTGCGAAAGC	FAM/ZEN/IowaBlack	250
β-actin forward	TTCAACACCCCWGCCATGTA	–	300
β-actin reverse	TCWCCGGAGTCCATCACRAT	–	600
β-actin probe	CCATCCAGGCYGTGCTGTCCCTG	VIC/ZEN/IowaBlack	250

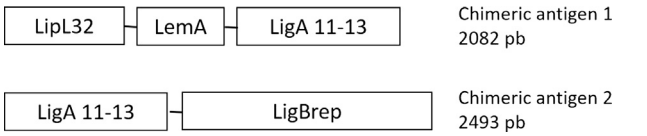

Fig. 1. Schematic illustration of chimeric proteins evaluated in this study, constructed from the combination of immunogenic antigens from *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130. Chimeric antigen 1 is a fusion of *lipL32* (24-272 aa), *lemA* (28-157 aa) and *ligANI* (943-1224 aa) and chimeric antigen 2 is a fusion of *ligANI* (943-1224 aa) and *ligBrep* (131–645 aa). *Figure adapted with permission from Carlos Eduardo Pouey da Cunha (manuscript submitted).*

selected in 7H10 medium with kanamycin and grown for 5 days in selective 7H9 medium. Expression of the recombinant proteins was confirmed by Western blot, using BCG cultures previously normalized by measuring optical density at 600 nm. Cells were then centrifuged at 4000*g* for 10 min, suspended in 1 ml of 100 mM Tris, pH 8.0, and disrupted using a Ribolyser (Hybaid, Kalletal, Germany). The total proteins were separated in SDS-PAGE 10% and electrotransferred to a nitrocellulose membrane (GE Healthcare, Illinois, United States). Blots were probed with mouse hyperimmune sera produced against each one of the recombinant chimeric proteins (1:500) and peroxidase-conjugated anti-mouse immunoglobulin G (Sigma-Aldrich, Missouri, United States) at a dilution of 1:4000. Detection was carried out using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Illinois, United States). Briefly, for production of hyperimmune sera, the recombinant chimeric antigens 1 and 2 (rQ1 rQ2) were produced in *E. coli* and purified as previously described [15]; then, two BALB/c mice of 6-week old were inoculated intraperitoneally with 100 μg of rQ1 or rQ2 plus Freund's incomplete adjuvant (Sigma Aldrich, Missouri, United States) on days 0, 14, 21 and 28. On day 35, blood was collected from retro-orbital venous plexus after administration of eye anesthetic drops, and samples were centrifuged and characterized regarding their ability to specifically recognize antigenic portions of chimeric proteins.

2.6. Vaccination and challenge of hamsters

Female and male hamsters aged between 4 and 6 weeks were randomly allocated into eleven groups of ten animals per group, equally distributed by gender, as described in Table 2. Animals were subcutaneously immunized with 10^6 CFU of rBCG strains with a 21-day interval (first dose at day 0 and second dose at day 21) between each immunization. A group of four hamsters was immunized in the quadriceps muscle with 10^9 heat-killed whole-leptospires as a positive control. Challenge was performed intraperitoneally fifty-one days after the first immunization (day 51) with a dose of 10^3 leptospires, equivalent to five times the 50% lethal dose (LD_{50}) of the *L. interrogans* sv. Copenhageni strain Fiocruz L1-130. Before each immunization and challenge, blood samples were collected from the retro-orbital venous plexus with administration of anesthetic eye drops, and the sera were stored at −20 °C.

Animals were humanly euthanized by deep anesthesia using pentobarbital when the end-point criteria were reached. These criteria consisted of loss of appetite, gait difficulty, prostration, dyspnea, ruffled fur, and weight loss of 10% of the animal's maximum weight [22]. Survivors were euthanized 30 days post challenge.

2.7. Humoral immune response determination

The antibody response was evaluated by indirect ELISA [15] using purified chimeric proteins. Each protein was used separately in a concentration of 500 ng per well, diluted in carbonate-bicarbonate buffer, pH 9.6. The ELISA plates were blocked with 5% fat-free dry milk, and hamster's sera were added at a 1:50 dilution in PBST (PBS with 0.05% [v/v] Tween 20) for 1 h at 37 °C. Anti-golden Syrian hamster IgG antibody conjugated to peroxidase (Rockland Immunochemicals, Pennsylvania, United States) was diluted (1:6000) and incubation proceeded at 37 °C for 1 h. Washing with PSBT was performed three times between all steps. Reactions were developed by adding o-phenylenediamine dihydrochloride (Sigma-Aldrich, Missouri, United States) and hydrogen peroxide and stopped with addition of 25 μl of 4 N H_2SO_4. Absorbance was read at 492 nm and mean values were obtained from serum samples assayed in triplicate.

2.8. Analysis of leptospiral presence in kidneys

The presence or absence of leptospires in the kidney samples of the surviving animals was evaluated by culture and real-time quantitative PCR (qPCR). Leptospiral genomic DNA in the kidney samples was quantified by qPCR using a LightCycler 96 system (Roche, Basel, Switzerland). Samples were prepared by dicing 100–200 mg of kidney tissue and suspending it in PBS, followed by tissue homogenization for two cycles of 20 s using a Ribolyser (Hybaid, Kalletal, Germany). Genomic DNA was extracted from approximately 40 mg of tissue using the SV Genomic DNA Purification kit (Promega, Brazil). Reactions were performed in triplicate using GoTaq Probe qPCR Master Mix (Promega) with the primers and probes described in Table 1. The cycling conditions were initial denaturation at 95 °C for 10 min, and 40 cycles of 15 s at 95 °C for

Table 2
Protection conferred by different rBCG-vectorized vaccines against lethal challenge in the hamster model of leptospirosis.

Immunogen[a]	% Protection (survivors/total)	*P* value[b]	Renal culture (positive/survivors)	qPCR (positive/survivors)
rBCG (pUP500/P_{pAN}:Q1)	100 (10/10)	< 0.0001	0/10	0/10
rBCG (pUP500/P_{18kDa}:Q1)	100 (10/10)	< 0.0001	0/10	0/10
rBCG (pUP500/P_{hsp60}:Q1)	100 (10/10)	< 0.0001	0/10	1/10
rBCG (pUP500/P_{ag85B}:Q1)	100 (10/10)	< 0.0001	0/10	0/10
rBCG (pUP500/P_{sag85B}:Q1)	80 (8/10)	0.0007	0/8	8/8
rBCG (pUP500/P_{pAN}:Q2)	60 (6/10)	0.0108	3/6	6/6
rBCG (pUP500/P_{18kDa}:Q2)	40 (4/10)	0.0867	3/4	4/4
rBCG (pUP500/P_{hsp60}:Q2)	40 (4/10)	0.0867	1/4	4/4
rBCG (pUP500/P_{ag85B}:Q2)	20 (2/10)	0.4737	0/2	2/2
rBCG (pUP500/P_{sag85B}:Q2)	20 (2/10)	0.4737	0/2	2/2
Bacterin (positive control)	100 (3/3)	0.0035	0/3	2/3
BCG Pasteur (negative control)	0 (0/10)	–	–	–

[a] Chimeric protein 1 is a fusion of genes *lipL32 (24-272 aa)*, *lemA (28-157 aa)* and *ligANI* (*943-1224 aa*); chimeric protein 2 is a fusion of genes *ligAni (943-1224 aa)* and *ligBrep (131–645 aa)*.
[b] Two-tailed *P* value determined by Fisher exact test in comparison to the negative control group.

denaturation and 60 s at 60 °C for annealing/elongation. The number of leptospires was determined in comparison to the number of hamster cells in the sample, quantified by copies of the *lipl32* and *β-actin* genes respectively. For culture, one kidney from each surviving animal was collected and inoculated in EMJH medium. During an eight-week incubation period, dark-field microscopy was performed to identify positive cultures.

2.9. Statistical analysis

Protection against mortality and the survival rates were determined using the Fisher's exact test and log-rank test respectively. Significant differences between the serological assays were determined by analysis of variance (ANOVA). $P \leq 0.05$ was considered to be statistically significant. Statistical analysis was carried out using the GraphPad Prism 7 and Statistix 8 software packages.

3. Results

3.1. Construction of rBCG expressing chimeric proteins of L. interrogans

Cloning of chimeric genes into pUP500 resulted in the construction of ten recombinant plasmids, named: pUP500/$_{pAN}$:Q1, pUP500/$_{18kDa}$:Q1, pUP500/$_{hsp60}$:Q1, pUP500/$_{ag85B}$:Q1, pUP500/$_{sag85B}$:Q1, pUP500/$_{pAN}$:Q2, pUP500/$_{18kDa}$:Q2, pUP500/$_{hsp60}$:Q2, pUP500/$_{ag85B}$:Q2 and pUP500/$_{sag85B}$:Q2. The expression of both chimeric proteins in rBCG under the control of five different mycobacterial promoters was evaluated by Western blot (Fig. 2). The polyclonal antibodies produced against each chimeric protein (anti-rQ1 or anti-rQ2) were able to specifically recognize their respective antigenic portions; i.e., LipL32, LigAni, LigBrep, and LemA (data not shown). Serum anti-rQ1 and anti-rQ2 were able to recognize chimeric proteins 1 and 2, with approximately 75 and 84 kDa respectively in rBCG cell lysates. No expression was detected in wild-type BCG extract used as negative control.

3.2. Antibody response elicited by rBCG vaccines

The antibody response induced by rBCG vaccines was evaluated by ELISA, and the results are summarized in Fig. 3. The humoral response induced by rBCG vaccines based on chimera 1 was significantly higher on day 21 than pre-immune day 0, and decreased on day 51. Significant antibody levels ($P \leq 0.05$) were observed in hamsters vaccinated with rBCG strains in which the expression of chimera 1 was controlled by promoters pAN, 18 kDa, and Ag85B plus signal sequence. Expression of chimera 1 in rBCG under the control of Hsp60 and Ag85B promoters did not induce significant levels of IgG ($P > 0.05$).

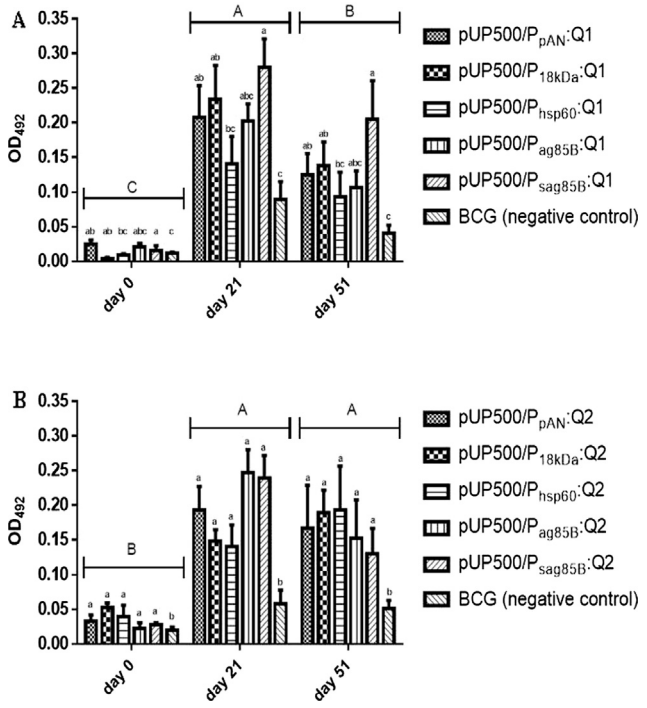

Fig. 3. IgG antibody response in hamsters immunized with rBCG vaccines. Purified recombinant chimeric proteins expressed by *E. coli* were used as the antigen in ELISA. (A) IgG levels induced by rBCG vaccines based on chimeric antigen 1 (Q1). (B) IgG levels induced by rBCG vaccines based on chimeric antigen 2 (Q2). The results represent mean absorbance ± standard deviation calculated from serum samples assayed in triplicate. OD_{492}, optical density at 492 nm. The significance was determined by the analysis of variance (Tukey multiple comparison). Groups without common lower case letters differ ($P < 0.05$); days without common upper case letters differ ($P < 0.05$).

All animals immunized with rBCG vaccines expressing chimera 2 produced significantly higher levels of IgG than those in the control group. However, no statistical difference was observed among vaccine groups. This response was maintained even after the second dose (day 51), and its magnitude was the same on day 21 ($P > 0.05$).

3.3. Protective efficacy of rBCG strains in hamster model

The immunoprotective potential of rBCG strains against leptospirosis was evaluated in terms of survival, recorded 30 days after challenge. The results of the mortality test (Fisher test) and the survival rates are shown in Table 2 and Fig. 4 respectively. All rBCG strains expressing the chimeric antigen 1 afforded 100%

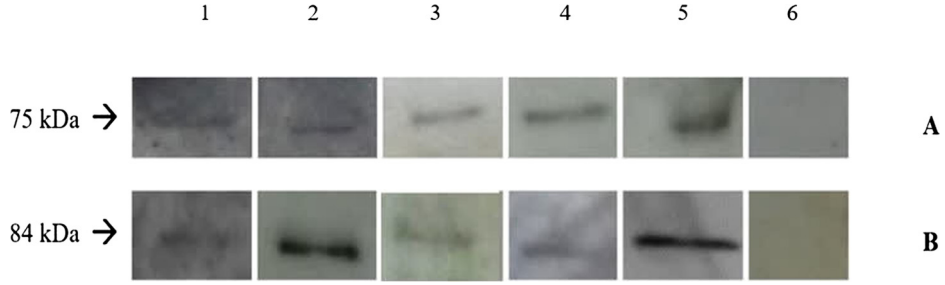

Fig. 2. Western blot demonstrating the expression of chimeric antigens by *M. bovis* BCG. Characterization of chimeric antigen 1 – Q1 (A) and 2 – Q2 (B) expressed in rBCG under control of five different mycobacterial promoters. Lane 1, rBCG transformed with pUP500/P$_{pAN}$:Q1 or Q2; lane 2, rBCG transformed with pUP500/P$_{hsp60}$:Q1 or Q2; lane 3, rBCG transformed with pUP500/P$_{18kDa}$:Q1 or Q2; lane 4, rBCG transformed with pUP500/P$_{ag85B}$:Q1 or Q2; lane 5, rBCG transformed with pUP500/P$_{sag85B}$:Q1 or Q2; lane 6, BCG Pasteur wild-type.

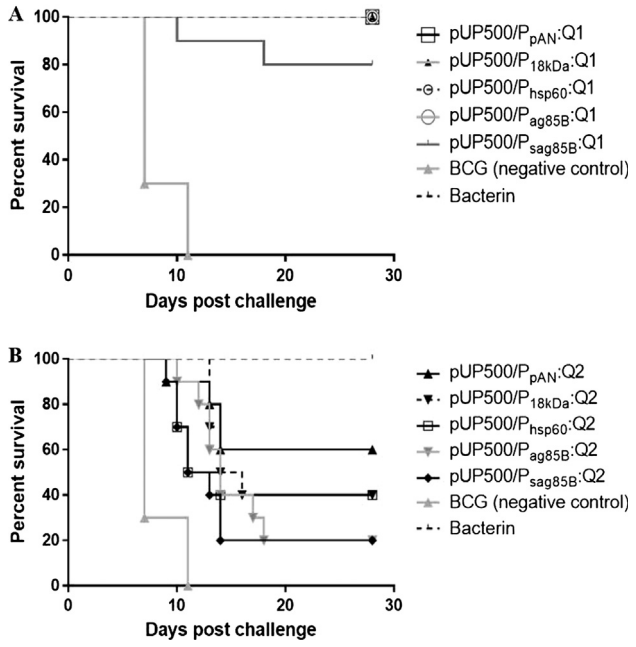

Fig. 4. Survival of hamsters immunized with rBCG after lethal challenge. Survival among hamsters vaccinated with rBCG strains or bacterin, after the administration of a lethal inoculum of *L. interrogans* serovar Copenhageni strain Fiocruz L1–130. (A) rBCG vaccines based on chimeric antigen 1 (Q1). (B) rBCG vaccines based on chimeric antigen 2 (Q2). Survival curves were compared using log-rank analysis.

protection against challenge except for the strain in which chimera 1 was secreted (Ag85B promoter plus signal sequence), which conferred 80% protection ($P < 0.05$). Among the rBCG strains expressing the chimeric antigen 2, only the one using the pAN promoter afforded significant survival rates after challenge (60% of survival). All animals vaccinated with bacterin survived after challenge. Endpoint criteria were observed in all animals from the negative control group, which received the wild-type BCG Pasteur.

3.4. Sterilizing immunity induced by rBCG vaccines

Bacterial culture and qPCR were used to evaluate whether vaccines were able to prevent renal colonization. All kidney cultures collected from the surviving animals immunized with rBCG strains expressing chimeric antigen 1 were negative. In qPCR, these vaccines did not prevent renal colonization in one animal from the group that received this antigen expressed under the control of Hsp60 promoter (1/10) and in all animals that received the construct using Ag85B promoter plus signal sequence (8/8). All survivors from the groups immunized with vaccines based on chimeric protein 2 were qPCR-positive, while results obtained by culture showed different rates of positive animals, ranging from 0% (pUP500/P$_{ag85B}$:Q2 and pUP500/P$_{ag85B}$:Q2 groups) to 75% (pUP500/P$_{18kDa}$:Q2 group). Animals from the negative control group were positive in culture (4/10) and in qPCR (9/10) (data not shown). Two of the three animals vaccinated with bacterin were also positive in qPCR (Table 2).

4. Discussion

Several proteins have exhibited potential as vaccine candidates against leptospirosis. The most evaluated antigens so far are the Lig proteins and the lipoprotein LipL32 [11]. Despite reports of protection, in most experiments, surviving animals remained chronic renal carriers. The hypothetical lipoprotein LemA also elicited partial protection against challenge, in prime-boost and DNA vaccination strategies, although the induction of sterilizing immunity has also not been reported [15]. Humoral immune response is known to be responsible for protection. However, in cattle, cellular immunity also plays a fundamental role in promoting protective immunity to *Leptospira* infection [23–25]. *M. bovis* BCG is a potent cellular immunity enhancer [17]. Thus, rBCG may represent an attractive vaccine vehicle for the expression of leptospiral antigens. Aiming to standardize the development of vectorized vaccines by BCG, we have recently developed a kit to work with mycobacteria according to BioBrick strategy [20] (data not published yet).

In this study, the expression of chimeric antigens containing sequences from *L. interrogans* in rBCG was achieved under the control of five different promoters using BioBrick vectors previously constructed by our group (data not published yet). Promoters evaluated in this study have been extensively used for expression of heterologous antigens in rBCG [19]. The first chimeric antigen includes amino acid sequences of LipL32, LemA, and LigANI, and was able to confer significant protection against challenge (80–100%) when expressed in rBCG under the control of any of the five promoters tested. Eighty percent of survival was observed when the Ag85B promoter plus a signal sequence was used. The reduction in this rate in comparison to the other groups might be explained by the secretion of the antigen to the extracellular medium, which may have affected protein conformation and immunogenicity [26]. The other chimeric antigen evaluated was a fusion of LigANI and LigBrep and was only able to protect hamsters when expressed under the control of pAN promoter. This could be attributed to the fact that this construction has a superior stability due to the characteristics of promoter, as demonstrated in other studies [27,28]. Both chimeric proteins were protective when evaluated as recombinant subunit vaccines; however, they did not confer sterile immunity (data not published). Using BCG as a vector, the chimeric antigen 1 presents a more effective combination of sequences to achieve protection than the second chimeric antigen. Recently, a subunit vaccine based on LigBrep was shown to be protective [14]. Surprisingly, the rBCG vaccines that afforded protection in the current study were predominantly based on chimeric antigen 1, which does not present LigBrep in its sequence. This might be due to the size of the antigens; chimeric antigen 2 presents a higher molecular weight, which may have been costly for its expression in BCG, reducing the bacterial fitness and resulting in the loss or truncated expression of the heterologous antigen and instability of the recombinant strain. rBCG instability expressing OspA has been considered a determinant for the lack of protection against *Borrelia burgdorferi* [29].

There is no immune correlate established for leptospirosis that can be associated with protection [30]. Here, we evaluated the humoral immune response by ELISA and observed significant levels of IgG induced by rBCG vaccines with only a single dose. Vaccines in which the promoters pAN, 18 kDa, and Ag85B plus a signal sequence commanded the expression of chimera 1 were able to induce a significant IgG response (Fig. 3, panel A). Chimera 2 was able to induce antibodies when expressed by any of the promoters evaluated; however, protection was only achieved with the pAN promoter. Despite antibody production, several recombinant subunit vaccines did not confer protection against leptospirosis [11]. Anti-LPS antibodies have been shown to be involved in protective immunity against infection; however, they are not sufficient to protect cattle, highlighting the role of cellular response in this context [11]. In light of its adjuvant potential, the use of BCG may represent a potential strategy by which it is possible to strengthen the immune response of recombinant antigens. Analysis of the cellular response will be of importance, especially when using BCG as a vaccine vector; however, this is hampered by the lack of knowl-

edge and molecular tools that are available to characterize hamster immunity.

It is well known that culture isolation is not the most reliable method of detecting the presence of leptospires in kidneys [31]. Thus, we also performed a quantitative real-time PCR using a specific probe for *lipL32* to evaluate the sterile immunity induced by rBCG vaccine preparations. We believe that the use of specific probes in qPCR is more reliable, sensitive, and specific than other methods such as imprint and qPCR based on SYBR green. The lack of a definitive and standardized tool to determine leptospiral colonization makes the association of different methods as the most rational approach to confirm bacterial burden in kidneys [32]. Until now, sterilizing immunity has been reported only for a LigBrep-based subunit vaccine [14].

Here, we demonstrated that four of the five rBCG vaccines based on chimeric protein 1 protected hamsters against challenge and renal colonization. This corroborates with other studies that have evaluated chimeras as vaccine antigens against leptospirosis [33–35]. A chimeric protein based on LigA, Mce, Lsa45, OmpL1, and LipL41 antigens was able to confer 50% of protection in hamsters [33]. In guinea pigs, Lin et al. [34] evaluated the immunoprotective potential of a chimera with epitopes from antigens OmpL1, LipL32 and LipL21, demonstrating 80% of survival compared to the control group immunized with PBS. A DNA vaccine based on LipL32 and LipL41 also demonstrated ability to reduce kidney colonization, however no protection was observed after challenge [35]. Our results highlight the potential of BCG as a vector to deliver *Leptospira* antigens and to construct chimeric genes as a promising alternative for the development of an effective and sterilizing vaccine against leptospirosis.

5. Conclusions

To the best of our knowledge, this study is the first of its kind to demonstrate the potential of chimeric antigens in live vaccines vectorized by BCG against leptospirosis. We also validated the application of BioBrick vectors in the expression of heterologous antigens in BCG, which can be expanded to other pathogens of interest. Further studies are necessary to investigate whether these vaccines are stable and able to protect animals against different serovars of *Leptospira* using a natural route of infection.

Authors contributions

Conceived and designed the experiments: TLO, CEPC, CR, OAD. Performed the experiments: TLO, CR, JD, ACPSN, MGA. Analyzed the data: TLO, CEPC, CR. Wrote the paper: TLO, DDH, OAD. Final approval of the version to be submitted: TLO, CEPC, CR, JD, ACPSN, MGA, DDH, OAD.

Conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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