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**Tese**

# Vacinas Recombinantes contra Leptospirose: construção e avaliação em modelos biológicos

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# Vacinas Recombinantes contra a Leptospirose: construção e avaliação em modelos biológicos

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## RESUMO

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A leptospirose é uma doença infecciosa causada por espiroquetas que pertencem ao gênero *Leptospira*, classificada como uma zoonose direta de ampla distribuição geográfica, que ocorre de forma endêmica no mundo inteiro. Os prejuízos na saúde pública e as perdas econômicas causadas por esta zoonose justificam o uso de vacinas em humanos e em populações de animais com risco. A proteína de membrana externa LipL32 foi selecionada para este trabalho, pois é conservada e encontrada somente em sorovares patogênicos de leptospira, apresentando grande potencial imunogênico. O gene *lip*L32 foi clonado no vetor de expressão em eucariotos pTarget (vacina de DNA); nos vetores pUS973, pUS974, pUS977 e pUS2000 para expressão em *Mycobacterium bovis* BCG; e no vetor pAE para obtenção da proteína recombinante em *Escherichia coli* (vacina de subunidade). Após a construção das vacinas, camundongos foram imunizados e a resposta imune foi avaliada por ELISA, western blotting e imunofluorescência. Dentre as vacinas estudadas a resposta imune teve níveis mais elevados que induziu a maior resposta imune foi a vacina de subunidade, porém com BCG recombinante (rBCG) o título de anticorpos ainda continuava em ascensão no final de 12 semanas de experimento. Os anticorpos presentes no soro dos animais vacinados foram capazes de reconhecer a proteína na membrana da leptospira através da técnica de imunofluorescência indireta. Um anticorpo monoclonal anti-LipL32 foi capaz de inibir o crescimento de Leptospiras *in vitro*, indicando o potencial imunoprotetor do antígeno LipL32. Hamsters imunizados com rBCG expressando LipL32 mostraram níveis significativos de proteção ($P \leq 0.05$) em desafio com um inóculo letal de *L. interrogans* serovar Copenhageni. Exames de autópsia não revelaram evidências macroscópicas ou histológicas da doença em hamsters imunizados com rBCG que sobreviveram ao desafio letal. Além disso, a seqüência que codifica os antígenos LipL32 e região não idêntica de LigA de *L. interrogans* foram clonados no sistema de expressão convencional em *M. bovis* BCG Pasteur e no sistema de seleção por

complementação auxotrófica em *M. bovis* BCG ∆*leu*D. A estabilidade dos vetores foi avaliada durante o crescimento *in vivo* do rBCG comparando seleção por complementação auxotrófica com seleção por resistência ao antibiótico. O sistema que utiliza complementação auxotrófica foi altamente estável *in vivo* enquanto o sistema convencional foi instável na ausência de pressão de seleção. Os resultados obtidos reforçam o potencial do antígeno LipL32 como um candidato promissor no desenvolvimento de vacinas contra a leptospirose, principalmente quando apresentado ao sistema imune de maneira adequada. Além disso, a estabilidade do BCG recombinante conferida pelo sistema de complementação auxotrófica pode melhorar a resposta imune induzida por este vetor vacinal, o qual pode ser utilizado para expressar antígenos de *Leptospira* e de outros patogenos.

**Palavras-chave:** leptospirose, proteína de membrana externa, vacina recombinante.

## ABSTRACT

SEIXAS, Fabiana Kömmling. **Recombinant Vaccines against Leptospirosis: construction and evaluation in biological models.** 2007. 109 f. Tese (Doutorado) - Programa de Pós - Graduação em Biotecnologia Agrícola. Universidade Federal de Pelotas, Pelotas.

Leptospirosis is an infectious disease caused by spirochetes belonging to the genus Leptospira, classified as a direct zoonosis with wide geographical distribution. The implications in terms of public health and economical losses caused by leptospirosis justify the use of a vaccine against *Leptospira* in human and animal populations at risk. In this study we used the external membrane protein LipL32 as a model antigen, as it is conserved among pathogenic species and highly immunogenic. The LipL32 coding sequence was cloned into several expression vectors: pTarget, to create a DNA vaccine; pUS973, pUS974, pUS977 and pUS2000 vectors for expression in *Mycobacterium bovis* BCG; and pAE to express the recombinant protein in *Escherichia coli*, for a subunit vaccine. Mice were immunized with the constructs and the immune response was evaluated by ELISA, immunofluorescence and Western blotting. The highest humoral immune response was elicited by the subunit vaccine (rLipL32). However, with recombinant BCG (rBCG) the antibodies titre was still rising at the end of the 12-week experiment. The antibodies of vaccinated animals were able to recognize LipL32 on the membrane of *Leptospira*, detected by indirect immunofluorescence. A monoclonal antibody anti-LipL32 was shown to inhibit the growth of *Leptospira in vitro*, indicating potential protection induced by the LipL32 antigen. Hamsters immunized with rBCG expressing LipL32 were protected against mortality ($P \leq 0.05$) upon challenge with a lethal inoculum of *L. interrogans* serovar Copenhageni. Autopsy examination did not reveal macroscopic or histological evidence of disease in rBCG immunized hamsters that survived lethal challenge. Moreover, the coding sequences for LipL32 and the non-identical fraction of LigA of *L. interrogans* were cloned and expressed in conventional *M. bovis* BCG Pasteur and *M. bovis* BCG Δ*leuD*, a novel system that allows selection by auxotrophic complementation. Stability of plasmid vectors was evaluated during *in vivo* growth of the rBCG in comparison to selection by antibiotic resistance. The new system was highly stable even during *in vivo* growth, as the selective pressure is maintained,

whereas in the conventional system, plasmid vectors were unstable in the absence of selective pressure. The data presented here further enhance the status of LipL32 as a promising candidate antigen for use in the control of leptospirosis, when presented to the immune system by an appropriate delivering system. In addition, the stability of recombinant BCG conferred by the auxotrophic complementation system may improve the immune response induced by this vaccine vector, which can be used to efficiently deliver not only leptospiral antigens, but also antigens from other pathogens.

**Key words:** leptospirosis, outer membrane proteins, recombinant vaccine

**SUMÁRIO**

# 1 INTRODUÇÃO GERAL

A leptospirose é uma doença infecciosa endêmica causada por espécies patogênicas de bactérias do gênero *Leptospira*, é classificada como uma antropozoonose direta de ampla distribuição geográfica, que ocorre de forma endêmica no mundo inteiro (BHARADWAJ, 2004). Dentro das diferentes espécies, encontram-se sorovares antigenicamente relacionados, que constituem os sorogrupos. Já foram descritos mais de 260 sorovares, distribuídos em 29 sorogrupos (FAINE et al., 1999). O número de sorovares e sorogrupos tende a aumentar, na medida em que novas técnicas, principalmente de biologia molecular, são desenvolvidas e/ou aprimoradas para tipificação dos isolados.

As leptospiras são transmitidas ao homem principalmente através da urina de diversas espécies de animais infectados (LEVETT, 2001). Os roedores são os principais reservatórios da doença, pois albergam a leptospira nos rins, eliminando-as vivas no meio ambiente e contaminando água, solo e alimentos. Dentre os roedores domésticos (*Rattus norvegicus, Rattus rattus* e *Mus musculus*), grande importância deve se dispensar ao *R. norvegicus*, portador clássico da *L. Icterohaemorraghiae*, a mais patogênica ao homem. A infecção humana pela leptospira resulta da exposição direta ou indireta à urina de animais infectados. Em áreas urbanas, o contato com águas e lama contaminados demonstra a importância do elo hídrico na transmissão da doença ao homem, pois a leptospira dele depende para sobreviver e alcançar o hospedeiro. Há outras modalidades de menor importância de transmissão como a manipulação de tecidos animais e a ingestão de água e alimentos contaminados. A transmissão de pessoa a pessoa é muito rara e de pouca importância prática. (FAINE et al., 1999).

As leptospiras penetram no hospedeiro através de pequenas escoriações ou abrasões na pele, ou ainda através da pele íntegra quando este permanecer por longos períodos em contato com águas contaminadas. Ao atingirem a corrente circulatória as leptospiras multiplicam-se rapidamente. A fase de bacteremia pode durar de 1 a 7 dias, e é concomitante com o aparecimento de sintomas como febre e dores musculares. Após, desaparecem completamente da circulação, constituindo assim, a fase imune. É nesta fase que há produção de anticorpos e eliminação das leptospiras pela urina (BAL, 2005; LEVETT, 2001). Desta forma a leptospirose se manifesta como uma enfermidade bifásica.

As manifestações clínicas da doença em 90% dos casos são benignas e autolimitadas (LEVETT, 2001), não permitindo uma distinção segura entre a leptospirose e inúmeras outras síndromes febris e miálgicas semelhantes. Os sintomas vão desde um estado febril moderado, com vômitos, dores de cabeça, musculares e abdominais, evoluindo em 5 a 15% dos casos a quadros mais severos de doença de *Weil*, caracterizada por falhas renais e do fígado ou leptospirose pulmonar, que acarreta hemorragia pulmonar fatal, com uma taxa de mortalidade de 5 a 40% (SEHGAL; MURHEKAR; SUGUNAN, 1995; FAINE et al., 1999; BHARTI et al., 2003). A maioria das complicações clínicas da leptospirose está associada, portanto, à localização das espiroquetas nos tecidos durante a fase imune.

O ressurgimento do interesse no controle da leptospirose deve-se a sua ocorrência como epidemia, tanto em países de clima tropical, subtropical ou temperado, desenvolvidos ou em desenvolvimento (BHARADWAJ, 2004). O Brasil e a China são os países em que a leptospirose é tida como um dos maiores problemas de saúde pública (MCBRIDE et al., 2005).

A leptospirose tem causado ainda impacto econômico para a agropecuária, com alta mortalidade nos rebanhos, abortos, natimortos, infertilidade e redução na produção de leite (BHARTI et al., 2003). Esses problemas resultam em graves prejuízos para os produtores e, conseqüentemente, para a economia dos países acometidos, pois causam transtornos produtivos e reprodutivos (FAINE et al., 1999).

Os prejuízos na saúde pública e as perdas econômicas causadas pela leptospirose justificam o uso de vacinas contra leptospirose em humanos ou em populações de animais com risco. O desenvolvimento de vacinas para prevenção da leptospirose humana tem sido reportado em alguns países. Vários experimentos encontram-se em fase pré-clinica e clínica, em Cuba (MARTINEZ et al., 2004) na Russia, (IKOEV et al., 1999) e China (ZHUO; WANG; LAN, 1995). Em Cuba foram vacinadas mais de 10.000 pessoas com uma bacterina, obtendo-se 78% de proteção (MARTINEZ et al., 2004). Já para o protótipo de vacina testado em humanos na China, crianças menores de 14 anos não apresentaram proteção (ZHUO; WANG; LAN, 1995).

Vários problemas confrontam o desenvolvimento de uma vacina para prevenir a leptospirose. As bacterinas conferem uma imunidade pouco duradoura (6 a 12 meses), são sorovares específicas, geralmente produzem uma baixa imunidade (PETERSEN et al., 2001) além disso, ainda não há conhecimento suficiente dos

mecanismos envolvidos na proteção contra leptospirose. Vacinação de animais como cães e bovinos pode prevenir enfermidade, mas não a leptospiruria e conseqüentemente a transmissão para humanos (BOLIN; ALT, 2001).

Atualmente é imprescindível o desenvolvimento de uma vacina protetora e de amplo espectro contra a leptospirose, através da identificação de antígenos protéicos conservados. Neste sentido, proteínas de membrana externa de *Leptospira* têm sido os maiores alvos para o desenvolvimento da vacina. Esforços para a identificação de componentes imunogênicos com potencial para o desenvolvimento de vacinas recombinantes resultou na caracterização das proteínas de membrana externa de 21, 31, 32, 36 e 41 kDa (HAAKE et al., 1993; SHANG et al., 1995; SHANG; SUMMERS; HAAKE, 1996; HAAKE et al., 1999; CULLEN et al., 2003). Recentemente uma nova família de proteínas (Ligs) foi descrita (PALANIAPPAN et al., 2002). Dados preliminares indicam seu potencial no desenvolvimento de vacinas recombinantes contra leptospirose, pois são proteínas de membrana externa presentes em todos os sorovares de leptospiras patogênicas e a expressão destas proteínas ocorre durante a infecção (MATSUNAGA et al., 2003; KOIZUMI; WATANABE, 2004).

Vários trabalhos têm sido publicados indicando o potencial da proteína LipL32, como sendo uma OMP (proteína de membrana externa), candidata ao desenvolvimento de uma vacina contra a leptospirose. Análises do perfil protéico das proteínas leptospirais indicam LipL32 como a proteína mais proeminente, sendo a principal proteína de membrana externa (ZUERNER et al., 1991). A seqüência e a expressão de LipL32 são altamente conservadas entre espécies de leptospiras patogênicas e está ausente em leptospiras não patogênicas (HAAKE et al., 2000). Além disso, LipL32 é expressa durante a infecção no hospedeiro, sendo altamente antigênica (HAAKE et al., 2000); >95% de pacientes com leptospirose produzem anticorpos contra LipL32 durante infecção (GUERREIRO et al., 2001). ZUERNER et al., 1991 demonstraram que a extração da membrana externa de leptospiras com os detergentes Triton X-100 ou Triton X-114 permitiu a solubilização da proteína de 32 kDa, LipL32, lipoproteína que representa o antígeno mais proeminente identificado por anticorpos de suínos infectados. Análises imunohistoquímicas de rins de hamsters infectados com *Leptospira kirschneri* demonstraram intensa reatividade com anticorpos anti-LipL32 (BARNETT et al., 1999). A chave para a antigenicidade de LipL32 está no seu alto nível de expressão em leptospiras patogênicas e nas

modificações lipídicas no aminoácido terminal cisteína (HAAKE et al., 2000). Como outras lipoproteínas bacterianas, LipL32 estimula a imunidade inata através da ligação e estimulação dos receptores CD14 e TLR2 (WERTS et al., 2001). O interesse em LipL32 como uma vacina deriva do achado que proteínas do extrato bruto de leptospiras com tamanho em torno de 31-34 kDa proporcionaram proteção cruzada em gerbils (SONRIER et al., 2000). Adenovírus recombinante e vacina de DNA tem sido utilizados para expressão de LipL32, conferindo proteção parcial contra desafio com uma cepa virulenta *de Leptospira interrogans* (BRANGER et al., 2001; BRANGER et al., 2005).

Nas últimas décadas, o rápido progresso das pesquisas, em particular nas áreas de imunologia e biologia molecular, lançou as bases para os avanços na tecnologia de produção de vacinas o que permitiu a introdução de novas estratégias para a obtenção e produção de antígenos, assim como foram otimizadas novas maneiras de se administrar e apresentar esses antígenos para as células do sistema imune (ADA; RAMSHAW, 2003).

Entre as possíveis estratégias para a prevenção da leptospirose a serem avaliadas temos vacina de subunidade, vacina de DNA e a utilização de *Mycobacterium bovis* BCG como vetor vacinal. As vacinas de subunidade têm o potencial de conferir proteção, sem o risco de causar a doença. Mais recentemente começaram a ser desenvolvidas vacinas de DNA. Estas também não apresentam risco de causar a doença, e tem a vantagem de possibilitar a indução de ambos os tipos de imunidade protetora, humoral e celular. Estas vacinas produzem a proteína dentro da célula, estimulando assim as duas classes do sistema MHC (BABIUK et al., 2000). Já a utilização de BCG como vetor de vacina oferece uma série de vantagens, tais como: é a vacina mais usada no mundo, pode ser ministrada em dose única após o nascimento, não sendo afetada pelos anticorpos maternos, é um importante adjuvante em experimentos animais, pode ser administrada via oral, é a vacina viva mais estável ao calor e seu custo de produção é baixo (OHARA; YAMADA, 2001). Nos últimos anos, sistemas para a manipulação e expressão de genes heterólogos em micobactérias foram desenvolvidos, permitindo a avaliação do BCG recombinante como um veículo para antígenos heterólogo de uma variedade de antígenos, revisado por (DENNEHY; WILLIAMSON, 2005). Nosso grupo tem demonstrado a utilização de BCG para a expressão de antígenos virais, bacterianos e parasitários (DELLAGOSTIN et al., 1993; DELLAGOSTIN et al., 1995; DA CRUZ et

al., 2001; MEDEIROS et al., 2002; BASTOS et al., 2003; MEDEIROS et al., 2005; MICHELON et al., 2006; SEIXAS et al., 2007).

Nesse contexto, a hipótese deste trabalho é que o antígeno LipL32 é capaz de induzir uma resposta imune específica com potencial imune protetor contra leptospirose, quando utilizado como vacina de subunidade, vacina de DNA ou vacina vetorizada por BCG recombinante. Assim, foram traçados os seguintes objetivos: (1) Avaliar a imunogenicidade do antígeno vacinal LipL32 sob diferentes formas de apresentação ao sistema imune; (2) Avaliar o potencial imunoprotetor do rBCG/*LipL32,* através de testes de desafio em hamsters com um inóculo letal de *L. interrogans* serovar Copenhageni e (3) Avaliar comparativamente a estabilidade *in vivo* do sistema de seleção e expressão por complementação auxotrófica em *M. bovis* BCG ∆*leu*D e do sistema de expressão convencional em *M. bovis* BCG Pasteur, utilizando antígenos de *Leptospira interrogans*.

Optamos por apresentar a tese na forma de artigos científicos. A nosso ver, essa modalidade é mais prática que o modelo de tese tradicional, uma vez que propicia uma divulgação objetiva e rápida dos resultados obtidos.

Inicialmente é apresentada uma revisão bibliográfica (artigo 1) sobre a utilização do *Mycobacterium bovis* BCG como vetor vacinal. Revisamos suas potencialidades e limitações, tanto na expressão de proteínas heterólogas de vírus, bactérias e parasitos, como na imunomodulação da resposta imune. Discutimos ainda a utilização de cepas de BCG auxotróficas, tema do quarto artigo desta tese. Esta revisão será submetida à publicação na revista **Vaccine**.

Em seguida, o artigo 2 trata da avaliação da capacidade do antígeno vacinal LipL32 de induzir uma resposta imune específica com potencial imune protetor contra leptospirose, quando utilizado como vacina de subunidade, vacina de DNA ou vacina vetorizada por BCG recombinante. Esse trabalho foi recentemente publicado no periódico **Canadian Journal of Microbiology**.

Na continuidade, o potencial imunoprotetor do antígeno LipL32 foi avaliado utilizando *M. bovis* BCG como vetor vacinal e hamsters como modelo animal para experimentos de desafios com cepa virulenta de *L. interrogans*. Os resultados mostraram que BCG expressando LipL32 é capaz de induzir uma resposta imune protetora em hamsters. Esse trabalho será submetido para publicação no periódico **Vaccine.**

No último artigo, foram comparadas a estabilidade *in vivo* do sistema de seleção e expressão por complementação auxotrófica em *M. bovis* BCG Δ*leu*D e do sistema de expressão convencional em *M. bovis* BCG Pasteur, utilizando os antígenos vacinais de leptospiras patogênicas, LipL32 e a fração não idêntica de LigA. Este trabalho será submetido na forma de *Short Communication* para a revista **FEMS Microbiology Letters**. Os artigos estão compilados na formatação exigida por cada um dos periódicos científicos em que foram ou serão publicados.

## 2 ARTIGO 1

**RECOMBINANT *Mycobacterium bovis* BCG: A REVIEW**

(Revisão a ser submetida ao periódico Vaccine)

# RECOMBINANT *Mycobacterium bovis* BCG

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**ABSTRACT**

BCG is an attenuated strain of *Mycobacterium bovis* that has been broadly used as a vaccine against human tuberculosis. This live bacterial vaccine has several advantages, however its efficiency in conferring protection against tuberculosis is a matter of considerable debate. BCG is able to establish a persistent infection and induces both cellular and humoral immune responses. The development of mycobacterial genetic systems to express foreign antigens and the adjuvanticity of BCG are the basis of the potential use of this attenuated mycobacterium as a recombinant vaccine. Over the years, a range of strategies has been developed to allow controlled and stable delivery of viral, bacterial and parasite antigens in BCG. Herein, we review the strategies developed to express heterologous antigens in BCG and the immune response elicited by recombinant BCG. In addition, the use of recombinant BCG as an immunomodulator and the future perspectives of BCG as a recombinant vaccine vector are discussed.

**Key words:** *Mycobacterium bovis* BCG, recombinant BCG, heterologous expression

## INTRODUCTION

The Baccillus Calmette-Guerin (BCG) is a strain of *M. bovis* that was empirically attenuated between 1906 and 1920 through several passages on glycerinated bile-potato medium. Subsequent studies inoculating BCG in mice, guinea pigs, calves, rhesus monkeys and chimpanzees demonstrated infectivity but complete attenuation of the strain. During *in vitro* passage, BCG underwent loss and/or rearrangement of several gene complexes that have been identified only recently [1-3]. In 1928, after experimental evaluations, BCG was recommended by the League of Nations as the official vaccine against human tuberculosis. Since then, it remains the only official and commercially available vaccine against tuberculosis (TB) [4]. BCG is currently the world's most widely used vaccine. It has been given to more than 3 billion people since 1921 [5]. BCG offers unique advantages as a vaccine since it is: (1) is unaffected by maternal antibodies and therefore it can be given at any time after birth; (2) is usually given as a single dose eliciting a long-lasting immunity; (3) stable and safe; (4) can be administrated orally; and (5) is inexpensive to produce when compared to other live vaccines. The extraordinary adjuvant properties of mycobacteria make them an attractive vector for the development of recombinant vaccines [6].

The interest in BCG has increased considerably since the last decade due to the development of genetic systems for expression of foreign antigens in mycobacteria. These systems include different shuttle-vectors, system to express and secrete heterologous antigens and strategies of mycobacteria transformation. Moreover, technologic advancement in the genomic of mycobacteria improved our understanding of the biology of this slow-growing pathogen and helped the conception of strategies for evaluation of BCG as a vaccine delivery vector [7]. Antigens of bacteria, parasites, and viruses have been expressed in this attenuated mycobacterium [8-11]. It has been shown that recombinant BCG (rBCG) elicits both cellular and humoral immune response against the heterologous antigens [12]. Recombinant BCG over expressing antigens of *M. tuberculosis* protects against tuberculosis better than the parental BCG strain [13, 14]

This review describes the systems available for heterologous antigen expression and compartmentalization in BCG. In addition, it is the immune events

elicited by recombinant BCG and the use of this attenuated strain as a recombinant immunomodulator are also discussed.

## BCG: A VECTOR TO EXPRESS HETEROLOGOUS ANTIGENS

In a pioneer study, Jacobs *et al*. [15] developed an *E.coli*-mycobacteria shuttle "plasmid-cosmid". In their work, a DNA sequence of the mycobacteriophage TM4, isolated from *M. avium*, was ligated to an *E. coli* cosmid and introduced into *E. coli*, *M. smegmatis* and BCG. The recombinant DNA molecule replicated in mycobacteria as a bacteriophage and in *E. coli* as a cosmid. The authors demonstrated not only the possibility of introducing exogenous DNA but also expressing heterologous genes in mycobacteria. However, the TM4-derived shuttle "plasmid-cosmid" was lytic for *M. smegmatis* and BCG, and therefore a new shuttle vector was constructed using the temperate mycobacteriophage L1 [16]. In addition, the aminoglycoside phosphotransferase gene was cloned into the temperate shuttle vector conferring stable kanamycin resistance upon transformation [16]. After that, several groups started working and improving the technology for expression of heterologous genes in BCG. Matsuo *et al*. [17] developed a system to express and secrete heterologous antigens from mycobacteria. Stover *et al.* [6] constructed plasmid vectors for expression of heterologous proteins in mycobacteria using the replication elements of the plasmid pAL5000 isolated from *M. fortuitum*. Dellagostin et al. [18] developed an integrative expression system containing two copies of the insertion sequence IS*900* from *M. paratuberculosis* flanking a kanamycin resistance gene in a "suicide" plasmid.

The expression of heterologous genes in BCG can be achieved using either replicating or integrating plasmids. Most of the mycobacterial replicative plasmids are designed using the origin of replication from the pL5000 that allows up to five copies of plasmid per transformants [19]. Therefore, one can expect to express a higher amount of recombinant protein using the replicative plasmids than the integrative vectors. However, it has been demonstrated that integrative vectors are more genetically stable both *in vitro* and *in vivo* than replicative plasmids [20].

Expression of foreign genes in mycobacteria can be modulated by the promoter used. Several promoters have been widely used in the available *E. coli*-mycobacterium shuttle vectors. The most frequently used are promoters from the

heat shock protein genes *hsp60* [6] or *hsp70* [21]. Other promoters used successfully in shuttle vectors include those from the *M. kansasii* (alpha) antigen [17], the *M. paratuberculosis* pAN [22], the *M. tuberculosis* 19 kDa antigen [23] and the *M. fortuitum* $\beta$ lactamase pBlaF* [24], as well as a compatible promoter from *Streptomyces albus*, GroES/EL1 [12]. Viral antigens have been expressed using *hsp60* and *hsp70* promoter, resulting in high-level of expression [23, 25, 26] and in some cases conferring protection [27]. The *Sm14* antigen of *Schistosoma mansoni* was expressed in *M. bovis* BCG under the control of the pBlaF* promoter, and this construction was shown to protect mice from cercarial challenge [28].

BCG was originally designed and tested by Calmette and Guerin to be an oral vaccine, and it is then logical to test recombinant strains of BCG by this route of administration [29]. Hayward *et al.* [30] described an oral vaccination of mice with rBCG expressing the B subunit of *E. coli* heat labile enterotoxin in the mycobacterial cytoplasm, cell wall and in a secreted form. The vaccinated animals developed IgG and IgA antibodies against the *E. coli* antigen, and the strongest response was found in mice vaccinated with rBCG expressing the heterologous antigen in a secreted form. In other study, BCG expressing HIV-1 envelope peptide V3 was tested as an oral vaccine in guinea pigs model. Peripheral blood mononuclear cells from immunized animals showed significant proliferative response [31]. Interestingly, delayed-type hypersensitivity against the HIV peptide was detected even 1.5 year after immunisation [31]. Méderlé *et al.* [32] immunized macaques with a single inoculation of three recombinant BCG strains expressing the SIVmac251 *nef*, *gag*, and *env* genes. The animals then received rectal and oral boosting doses of the rBCGs. The authors showed that the mucosal booster dose increased production of IFN-$\gamma$ and induced production of mucosal IgA.

It has been showed that the dose of BCG is crucial in determining the Th1/Th2 nature of the immune response, with low doses favoring a predominantly cell-mediated Th1 response [33]. Based on that, it is possible to use a specific dose of rBCG to improve the chance to obtain a desirable immune response. A high antibody response was elicited inoculating mice with $10^8$ CFU of rBCG expressing the B subunit of human *E. coli* heat labile enterotoxin [30]. Pym *et al.* [34] reported that $10^6$ CFU of rBCG expressing ESAT-6 elicited a strong cellular immunity and, consequently protection against tuberculosis in mouse and guinea pig models.

It has long been known that the adjuvant activity of BCG resides in the mycobacterial cell wall. In order to increase the immunogenicity against foreign antigens expressed in BCG, there may be advantage to use mycobacterial signal sequences to drive the expression to the bacterial wall. Therefore, the MT19 signal sequence, from *M. tuberculosis*, has been used to drive the heterologous antigen to the mycobacterial surface [23, 25, 27]. Immunization of mice with rBCG expressing the outer surface protein A of *Borrelia burgdorferi* as a membrane-associated lipoprotein resulted in protective antibody response that was 100-1000-fold higher than the response elicited by immunization with rBCG expressing the same antigen expressed cytoplasmically or as a secreted fusion protein [23]. Bastos *et al.* [27] vaccinated mice with BCG expressing glycoprotein 5 and matrix protein of porcine reproductive and respiratory syndrome virus either in the cytoplasm or on the mycobacterial membrane. In this study, neutralizing antibodies against the virus were only detected in mice inoculated with rBCG expressing the viral antigens on the mycobacterial surface, suggesting an improvement of antigen presenting and induction of humoral immune response. In some cases, it is also interesting to include mycobacterial signal sequences that allow a significant number of recombinant proteins to be secreted from rBCG [30]. The alpha antigen, from *M. kansaii*, has been widely used as a signal sequence to secret foreign antigens from rBCG [17]. Langermann *et al.* [25] showed protective humoral response in mice vaccinated with rBCG expressing pneumococcal surface protein A on the mycobacterial surface. However, no protection was induced in animals vaccinated with rBCG expressing the same pneumococcal antigen in cytoplasm, suggesting a betterment of immunogenicity, when the antigen is expressed in the membrane of BCG.

## BCG: A RECOMBINANT VACCINE

Live bacterial vaccines in general require no additional adjuvant component to evoke immune response in several animal models [35]. For BCG, usually a single inoculation is sufficient for induction of immune response and eventually protection. BCG is a superb delivery vector for heterologous antigens due to its capacity of intracellular replication in antigen presenting cells, such as macrophages and dendritic cells [36]. The induction of immune response against heterologous

antigens, following inoculation with rBCG was initially reported by Stover *et al.* [6] and Aldovini & Young [21]. Stover *et al.* [6] tested integrative and multicopy plasmid systems to express heterologous antigens in BCG. Using these two systems, β-galactosidase, tetanic toxin and HIV-1 antigens were expressed in BCG under the control of the mycobacterial *hsp*60 and *hsp*70 gene promoters. Aldovini & Young *et al* [21] utilized a multicopy plasmid system to express HIV-1 proteins in BCG under control of the mycobacteria *hsp*70 gene promoter. Both studies report the development of humoral and cellular immunity against the heterologous antigens following inoculation of the rBCG vaccines in mouse model.

The first evidence of protective immunity elicited by rBCG was demonstrated by Stover *et al.* [23]. In that study, protective humoral immune response was induced in mice inoculated with rBCG expressing *Osp*A antigen from *Borrelia burgdorferi*. Despite of the protection induced by rBCG-OspA in mouse model [23], this recombinant strain failed to elicit protection against Lyme disease in humans [37]. This recombinant vaccine had a safety profile and the volunteers converted positive in the PPD test as expected, but it did not elicit a primary humoral response. Possible explanations to the rBCG-*Osp*A failure in humans is the small amount of *Osp*A production or the loss of the plasmid vector containing the *Osp*A gene [37]. BCG expressing LCR1 antigen of *Leishmania chagasi* induced protective immunity in susceptible mice [38]**.** BCG expressing glycoprotein 5 and matrix protein of porcine reproductive and respiratory syndrome virus elicited a degree of protection in swine [27]. Effective protection against cutaneous leishmaniasis was obtained in mouse model using rBCG expressing the leishmania surface proteinase gp63 [39]. Protective immune response against experimental *M. leprae* infection in mice was induced by rBCG over-expressing three components of mycobacterial Ag85 antigen [40]. Immunisation with recombinant BCG expressing the cottontail rabbit papillomavirus (CRPV) L1 gene also provides protection from CRPV challenge [41].

Recombinant BCG has been used to improve the efficacy of the BCG as a vaccine for tuberculosis, in some cases with better results than the parental vaccine. A recent study with guinea pigs immunized with rBCG30 and challenged with highly virulent *M. tuberculosis* by aerosol, had significantly less organ pathology, significantly fewer organisms in their lungs and spleen, and significantly greater survival than guinea pigs immunized with the parental strain of BCG. This recombinant BCG vaccine was the first vaccine reported to induce greater protective

immunity against TB than the standard BCG vaccine in an animal model. A phase I clinical trial with rBCG30 was started in 2004 [42]. In another study, the BCG was equipped with the membrane-perforating listeriolysin (Hly) of *Listeria monocytogenes,* which was shown to improve protection against *M. tuberculosis.* Following aerosol challenge, the Hly-secreting recombinant BCG vaccine was shown to protect significantly better against aerosol infection with *M. tuberculosis* than did the parental BCG strain [43].

Construction of multivalent recombinant BCG is a goal that has been permed for many scientific groups. Abomoelak *et al.* [44] expressed in BCG strains a pertussis toxin-tetanus toxin. The recombinant strains induce neutralizing antibodies against both toxins in the mouse model. Mederle *et al.* [20] constructed multivalent rBCG strains by using integrative vectors expressing *nef* and *gag* of simian immunodeficiency virus. The strains were highly stable *in vivo* and induced a longer antibody response than non-integrative strains. In addition, the administration of rBCG “cocktails”, containing more than one recombinant strain, has also been shown to elicit immune response and some level of protection against the heterologous antigens in different animal models [27, 45]. It has been demonstrated that expression of heterologous antigens in the mycobacterial cytoplasm, on the mycobacterial surface, or in a secreted form may influence the immune response, and consequently the protective immunity induced against heterologous antigens expressed in BCG. Langerman *et al.* [46] described the development of specific humoral immune response in mice induced by rBCG expressing the *Psp*A antigen from *Streptococcus pneumoniae*. However, only the strain expressing the antigen on the BCG surface and in a secreted form elicited protection.

Despite of the success of heterologous antigen expression and, in some cases, protection induced by rBCG, *in vitro* and *in vivo* instability of the recombinant vaccine has been reported [47]. This instability is reported mainly with replicative vectors that are lost during BCG replication *in vivo* [37]. Vectors that integrate into the BCG genome are more stable [48], however, the disadvantage is the lower expression level of heterologous genes compared to that of multicopy plasmids. Thus, optimization of BCG as a vehicle for live recombinant vaccines requires improved strategies for stable antigen expression. We are currently involved in the construction of new *M. bovis* BCG expression vectors using auxotroph strain of *M. bovis* BCG and auxotrophic complementation as selectable marker. Preliminary

results indicate that this selectable system is stable *in vivo*, as the selective pressure is maintained. Several bacterial antigens are being used to evaluate this system.

Tables 1, 2, and 3 present a detailed list of heterologous bacterial, parasite, and viral antigens expressed in BCG, as well as the promoter used, route and dose administered, site of expression, immune response elicited and animal model used.

## RECOMBINANT BCG: AN IMMUNOMODULATOR

Immunomodulators are substances or live organisms that accelerate, prolong, or enhance the quality of specific immune response to antigens. Live, attenuated, recombinant bacterial vaccines have been largely used to modulate the immune system to respond in a specific profile to a specific antigen [49].

The use of a recombinant BCG expressing IFN-γ (BCG-IFN) resulted in an alteration in the pattern of inflammation and local tissue fibrosis. These results demonstrated that granulomas in the areas of mycobacterial infection are active sites of both inflammation and fibrosis. In addition, local expression of IFN-γ by recombinant BCG results in more efficient bacterial clearance, which is accompanied by a reduction in tissue pathology [50].

The immunomodulator effect of wild type BCG (wtBCG) has been well described [51, 52], and recently the expression of cytokines has even improved this effect. This approach has allowed modulation of the immune system to respond with a specific and desired pattern of cytokines [53]. Young *et al.* [54] demonstrated that rBCG secreting IL-2 induced a strong type 1 immune response in mice. It also induced an antibody isotype shift characterizing a type 1 immune response. However, the authors found that lymphocytes of mice vaccinated with rBCG expressing IL-18 produced significantly less IFN-γ than animals vaccinated with wtBCG. This impaired induction of IFN-γ was correlated to a significantly lower protection against *M. bovis* challenge. In contrast, Biet *et al* [55] demonstrated that rBCG producing IL-18 increased antigen-specific IFN-γ production in mice. Their results showed that the production of IL-18 by rBCG enhanced the immunomudulatory properties of BCG toward a Th1 profile and may be an alternative to treat bladder cancer in human patients. In addition, Luo *et al.* [56] demonstrated

that rBCG expressing IL-18 enhanced the Th1 immune response and that such strain of BCG may be used as an agent for bladder cancer immunotherapy.

The knowledge of the mechanisms used by microorganisms and cancer disorders to cause disease, combined with the events that correlate with immune protection, has been used to understand diseases and design more efficient vaccines. However, infections such as tuberculosis and HIV-AIDS among others, are still a challenge for the scientific community. In this context, we are looking forward to seeing a better understanding of the relationship between microorganisms and host in order to support the use of wtBCG and rBCG as immunomodulators to induce protection.

Table 4 present a detailed list of immunomodulators expressed in BCG, as well as the promoter used, route and dose administered, site of expression, immune response elicited and animal model used.

## RECOMBINANT BCG: PERSPECTIVES AND CONCLUSIONS

This review described recent progress that has been made towards understanding the recombinant BCG as a vaccine to protect against differents diseases. Many foreign antigens have been expressed in BCG and showed to provide adequate immune response and protection. However, a number of factors have been identified as important in the induction of immune response against the foreign antigen, for instance, dose, route, strain, antigen display and stability of the rBCG vaccine.

BCG is a good example of an “old fashion” attenuated vaccine that has been improved by molecular biology techniques. Recombinant BCG has been experimentally tested to elicit immune response and used to induce protection against several infectious diseases. It has been more than 70 years since BCG was officially recommended as a vaccine against TB, and today it still plays an important role in immunization against this disease and it certainly has an undoubted importance as a recombinant vaccine. Over the last 18 years rBCG with enhanced immunostimulatory properties have been genetic engineered and tested. Improvements in vector stability and selection systems have the potential of further improve rBCG not only as a vaccine against tuberculosis, but also against many other diseases, making it a truly multivalent vaccine.

**TABLE 1.** Bacterial antigens expressed in *Mycobacterium bovis* BCG.

| antigen (organism) | promoter | display expression | route | dose (CFU) | immunity | model | reference |
|---|---|---|---|---|---|---|---|
| *Lac*Z (*E. coli*) | *hsp*60 | C | IV,ID,IP | $2x10^6$ | HI,CI | mice | [6] |
| α-galactosidase (*E. coli*) | $P_{AN}$ | C | SC | $10^7$ | HI,IC | mice | [22] |
| *osp*A (*B. burgdorferi*) | *hsp*60 | C,M,S | IP | $10^6$ | HI | mice | [23] |
| *osp*A (*B. burgdorferi*) | *hsp*60 | C | IN,IP | $2x10^8$ | HI | mice | [58] |
| *psp*A (*S. pneumoniae*) | *hsp*60 | C,M,S | IP | $10^6$ | HI | mice | [36] |
| pertusis and tetanic toxin | *ag85a* | C,S | IV,IP | $5x10^6$ | HI,CI | mice | [46] |
| enterotoxin (*E. coli*) | *hsp*60 | C,M,S | IP,O | $10^8$ | HI | mice | [30] |
| *osp*A (*B. burgdorferi*) | *hsp*60 | S | ID | $10^7$ | HI | human | [39] |
| S1 toxin (*B. pertussis*) | *pBla*F* | C | IP | $10^6$ | HI,CI | mice | [59] |
| Antigens of *M. leprae* | *ag85b* | S | ID | $10^7$ | CI | mice | [42] |
| ESAT6, 19KDa, 38KDa (*M. tuberculosis*) | *Trrn* | C,S | IV | $10^6$ | HI,CI | mice | [60] |
| Cholera toxin B subunit | *hsp*60 | S | IN | $5x10^6$ | HI | mice | [56] |
| ESAT-6, CFP-10 (*M. tuberculosis*) | *Trrn* | S | SC | $10^6$ | CI | mice, guinea pig | [35] |
| ESAT-6 (*M. tuberculosis*) | *esat-6* | C,S | SC | $10^6$ | HI,CI | mice | [61] |
| FC (tetanus toxin fragment) | pBlaF* | C | IP | $5×10^6$ | HI, CI | mice | [62] |
| Ag85B (*M. tuberculosis*) | *S16* | S | IV | $10^6$ | HI,CI | mice | [63] |
| RD1 antigens | *Trrn* | S | IV | $10^6$ | CI | mice | [64] |
| S1 toxin (*B. pertussis*) | $P_{AN,}$ *hsp60* | C | IP | $10^6$ | HI | mice | [65] |
| 30 kDa (*Mycobacterium tuberculosis*) | *hsp60* | C | ID | $10^3$ | HI, CI | guinea pig | [66] |
| Ag85B-ESAT6 | *Hsp60* and a-ss signal sequences | C,S | SC | $10^6$ | HI,CI | mice | [67] |
| LipL32 (*Leptospira interrogans*) | $P_{AN,}$ *hsp60* | C,M | IP | $10^6$ | HI | mice | [68] |

*hsp*: heat shock protein. C: cytoplasm. M: membrane surface. S: secreted. IV: intravenous injection. ID: intradermal injection. IP: intraperitoneal injection. SC: subcutaneous injection. IN: intranasal. O: oral. IM: intramuscular CFU: colony-forming unit. HI: humoral immunity. CI: cellular immunity.

**TABLE 2.** Parasite antigens expressed in *Mycobacterium bovis* BCG.

| antigen (organism) | promoter | display expression | route | dose (CFU) | immunity | model | reference |
|---|---|---|---|---|---|---|---|
| gp63 (*Leishmania spp*) | *hsp*60 | C | SC,IV | $10^6$ | HI | mice | [41] |
| S-transferase (*S. mansoni*) | *hsp*60 | C,S | IV,IP,SC | $10^6$ | HI | mice | [69] |
| *gra*1 (*T. gondii*) | 85A | C,S | IP | $5x10^6$ | HI,CI | mice and lamb | [70] |
| *lcr*1 (*L. chagasi*) | *hsp*60 | C | IP,SC | $10^6$ | HI,CI | mice | [40] |
| Surface antigens (*P. falciparum*) | *hsp*70 | C | SC | $10^7$ | HI,CI | mice | [71] |
| Sj26GST (*Schistosoma japonicum)* | *hsp70* | C | - | - | HI,CI | mice | [72] |
| *Sm14 (Schistosoma mansoni*) | *pBlaF** | M | IP | $10^6$ | HI,CI | mice | [28] |
| *msp1A (Anaplasma marginale)* | *pBla*F*, 18kDa, | C | IP | $10^6$ | HI,CI | mice | [73] |
| Malarial epitopes F2R(II) EBA and (NANP)3 | *PAL500* | - | IP | $10^6$ | HI,CI | mice | [74] |
| ROP2 (*T. gondii*) | *hsp60* | C | - | - | HI,CI | mice | [75] |

*hsp*: heat shock protein. C: cytoplasm. M: membrane surface. S: secreted. IV: intravenous injection. ID: intradermal injection. IP: intraperitoneal injection. SC: subcutaneous injection. IN: intranasal. O: oral. IM: intramuscular CFU: colony-forming unit. HI: humoral immunity. CI: cellular immunity.

**TABLE 3.** Viral antigens expressed in *Mycobacterium bovis* BCG.

| antigen (organism) | promoter | site | route | dose (CFU) | immunity | model | reference |
|---|---|---|---|---|---|---|---|
| *gag*, *env,* and p*ol* (HIV-1) | *hsp*70 | C | ID | $5x10^6$ | HI,CI | mice | [21] |
| *nef* (HIV-1) | *groES* | C | SC | $10^7$ | CI | mice | [76] |
| *gag* (SIV) | *hsp70* | C | ID | $10^8$ | CI | macaque | [77] |
| *nef* (SIV) | $P_{AN}$ | C | SC | $10^7$ | HI,CI | mice | [78] |
| L1 e E7 (papilomavirus) | *hsp*70 | C | IV,SC,IN | $10^6$ | HI,CI | mice | [79] |
| *nef*, g*ag,* and e*nv* (SIV) | *hsp*70 | C | SC,IV | $10^8$ | HI,CI | macaque | [47] |
| Epitopes of rabies vírus | 18kDa, *hsp*60 | C | IP | $10^6$ | HI | mice | [80] |
| GP5 and M protein (PRRSV) | *hsp*60 | C,M | IP | $10^8$ | HI,CI | mice | [81] |
| V3 (HIV-1) | *hsp60* | - | SC,O | 320 mg | HI,CI | guinea pigs | [31] |
| Nef, gag, env (SIV) | $P_{AN}$ | C | ID,O,R | $5x10^8$ | CI | monkeys | [33] |
| CTL epitope (HCV) | - | S | IP | $2x10^7$ | CI | mice | [82] |
| GP5 and M protein (PRRSV) | hsp60 | M | IM | $10^8$ | HI,CI | swine | [27] |
| S genes (Hepatitis virus) | $P_{AN}$ | C | IP | $10^7$ | HI | mice | [83] |
| Gag (SIV) | hsp60 | C | ID | $10^7$ | CI | macaques | [84] |
| p24 Gag (HIV-1) | hsp60 | C | ID | $10^7$ | HI,CI | mice | [26] |
| Env V3 (HIV) | *hsp60* | - | - | - | - | macaques | [85] |
| I1 (CRPV) | *mtrA* | S | SC | $10^7$ | HI, CI | rabbits | [43] |
| p27Gag (SIV) | hsp60 | C | ID, O | 80-160mg | HI, CI | guinea pigs | [86] |
| VP6 (rotavirus) | *hsp60* | C,M,S | IP | - | HI,CI | mice | [87] |
| Glycoprotein Si (Infectious bronchitis virus IBV) | *hsp70* | M | IP | $10^6$ | - | mice | [88] |
| Env (HIV) | - | C,M,S | - | - | CI | mice | [89] |
| papillomavirus (CRPV) | *mtrA* | S | SC | $10^7$ | - | rabbits | [90] |

*hsp*: heat shock protein. C: cytoplasm. M: membrane surface. S: secreted. IV: intravenous injection. ID: intradermal injection. IP: intraperitoneal injection. SC: subcutaneous injection. IN: intranasal. O: oral. IM: intramuscular. R: rectal. CFU: colony-forming unit. HI: humoral immunity. CI: cellular immunity. HIV: human immunodeficient virus. SIV: simian immunodeficient virus PRRS: Porcine reproductive and respiratory syndrome. HCV: hepatitis C virus.

**TABLE 4.** Cytokines expressed in *Mycobacterium bovis* BCG.

| cytokine | Animal Model | immunological effect | reference |
|---|---|---|---|
| IL-2 | Mouse | efficient cytotoxicity, cytokines such as IL-12, tumor necrosis factor and interferon (IFN)-gamma | [91] |
| IL-2 andIL-18 | Mouse | T helper 1-type immune response | [92] |
| IL-18 | Mouse | IFN-γ production | [56] |
| IL-18 | Mouse | T helper 1-type immune response | [57] |
| IL-12 | Mouse | - | [93] |
| IFNgamma | Murine | up-regulated expression of MHC class I molecules | [94] |
| IL-18 and IL-5 | Mouse | Th2 response | [95] |

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# 3 ARTIGO 2

## EVALUATION OF DIFFERENT WAYS OF PRESENTING LIPL32 TO THE IMMUNE SYSTEM WITH THE AIM OF DEVELOPING A RECOMBINANT VACCINE AGAINST LEPTOSPIROSIS

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# EVALUATION OF DIFFERENT WAYS OF PRESENTING LIPL32 TO THE IMMUNE SYSTEM WITH THE AIM OF DEVELOPING A RECOMBINANT VACCINE AGAINST LEPTOSPIROSIS

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## ABSTRACT

Leptospirosis, caused by bacteria of the genus *Leptospira*, is a direct zoonosis with wide geographical distribution. The implications in terms of public health and the economical losses caused by leptospirosis justify the use of a vaccine against *Leptospira* in human or animal populations at risk. In this study we used the external membrane protein LipL32 as a model antigen as it is highly immunogenic. The LipL32 coding sequence was cloned into several expression vectors: pTarget, to create a DNA vaccine; pUS973, pUS974 and pUS977 vectors for expression in BCG (rBCG); and pAE to express the recombinant protein in *E. coli*, for a subunit vaccine. Mice were immunized with the various constructs and the immune response was evaluated. The highest humoral immune response was elicited by the subunit vaccine (rLipL32). However, with rBCG the titre was still rising at the end of the experiment. The serum of vaccinated animals was able to recognize LipL32 on the membrane of the *Leptospira*, detected by indirect immunofluorescence. A monoclonal antibody anti-LipL32 was shown to inhibit the growth of *Leptospira in vitro*, indicating potential protection induced by the LipL32 antigen.


**Key-words:** *Leptospira*, LipL32, recombinant BCG, subunit vaccine, DNA vaccine

# INTRODUCTION

Leptospirosis, a worldwide zoonotic infection with a high incidence rate in tropical regions, is classified as an emerging infectious disease (McBride et al. 2005). Transmission to humans occurs either through direct contact with an infected animal or through indirect contact via soil or water contaminated with urine from an infected animal (Faine 1982). There are more than 230 serovars among pathogenic leptospiras. The local variability in serovars of endemic leptospiral strains complicates the development of a vaccine that could be used worldwide (Levett 2001). Therefore, the development of new strategies for the prevention of leptospirosis is necessary. Immunogenic proteins, especially the outer membrane surface proteins, of pathogenic *Leptospira,* may be effective vaccinogens. LipL32, also called haemolysis associated protein-1 (Hap-1), is a promising vaccine candidate. It is the major outer membrane protein and is surface exposure (Cullen et al. 2005). Antigenicity of LipL32 was first identified in naturally infected dogs (Gitton et al. 1994), and it was demonstrated to be expressed during infection in hamsters (Haake et al. 2000). Subsequently, it was demonstrated that over 95% of human patients with leptospirosis produce antibodies to LipL32 during infection (Flannery et al. 2001). The nucleotide sequence coding for LipL32 is conserved among pathogenic Leptospira, whereas it is absent in nonpathogenic *Leptospira* (Haake et al. 2000). Vaccination using an adenovirus vector encoding the *lipL32/hap-1* gene induced cross-protection in the gerbil model of leptospirosis (Branger et al. 2001). The *lipL32/hap-1* gene derived from *L. interrogans* serovar Autumnalis provided protective immunity against a challenge with an heterologous strain of *L. interrogans* serovar Canicola (Branger et al. 2005).

Possible strategies for developing LipL32 into a recombinant vaccine against leptospirosis include its use as a subunit vaccine, as a DNA vaccine, or the use of a vaccine vector, such as *Mycobacterium bovis* BCG as a carrier for the antigen. BCG offers unique advantages as a vaccine: it is unaffected by maternal antibodies and therefore it can be given at any time after birth; BCG is usually given as a single dose eliciting a long-lasting immunity; it is stable and safe; BCG can be administrated orally; and it is inexpensive to produce when compared to other live vaccines (Ohara and Yamada 2001). Subunit vaccines have the potential of inducing protection

without the risk of causing the illness, whereas DNA vaccines are simple to make and deliver and can elicit both humoral and cellular immunity (Shams 2005).

The purpose of the present study was to investigate the immunogenicity of the LipL32 antigen presented to the immune system under different forms. We evaluated LipL32 as a subunit vaccine, as a DNA vaccine and as a live recombinant BCG vaccine expressing the LipL32 antigen.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Leptospiras were grown at 30°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with *Leptospira* Enrichment EMJH (Difco, USA). *Escherichia coli* strains DH5α and BL21(DE3) pLysS were grown in Luria-Bertani (LB) medium at 37°C with the addition of the appropriate antibiotic (kanamycin 50 µg/ml or ampicillin 100 µg/ml). *Mycobacterium bovis* BCG Pasteur strain 1173P2 was cultivated in stationary 25-$cm^2$ tissue culture flasks or in 50 ml tubes with agitation of 250 rpm at 37°C containing Middlebrook 7H9 (Difco) liquid medium supplemented with oleic acid-albumin-dextrose-catalase (OADC - Difco), 0.05% of Tween 80 and 0.2% of glycerol.

### DNA extraction, PCR amplification and cloning

The genomic DNA from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was prepared according to (Sambrook and Russel 2001). The *lipL32* gene, excluding the signal peptide coding sequence, was amplified by PCR using the oligonucleotide primers listed in table 1. PCR was carried out with the following program: 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec, with a final extension of 72°C for 7 min. The reactions were performed in final volume of 25 µl containing 2.5 µl of 10 × buffer, 0.5 µl of 10 mM dNTP, 150 ng of each primer, 0.5 µl (1 unit) of Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, USA), 0.5 µl of 50 mM $MgCl_2$, 18 µl of Milli-Q water and 2 µl of containing 50 ng of template DNA, in an Eppendorf Mastercycler thermocycler. The PCR products were subsequently cloned into vectors listed in table 1. All cloning

procedures were carried out according to standard procedures (Sambrook and Russel 2001).

**DNA Vaccine**

The plasmid used for nucleic acid immunization was the pTARGET™ expression vector (Promega, USA). The PCR amplified *lipL32* DNA fragment was cloned into pTARGET™, resulting in the plasmid named pTarget/*lipL32*. A colony of *E. coli* DH5α containing the recombinant plasmid was cultured in Luria-Bertani broth containing ampicillin. Large-scale plasmid DNA isolation was performed using the Perfectprep® Plasmid Maxi kit according to the manufacturer's directions (Eppendorf, Germany). The DNA was finally resuspended in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml. DNA concentration and purity were determined by optical density, and the $A_{260}/A_{280}$ ratio was typically greater than 1.9. The pTarget/*lip*L32 plasmid was verified by restriction digestion and by sequencing of the entire insert with an automated DNA sequencer (MegaBACE - Amersham Biosciences, USA).

**Expression and purification of recombinant $His_6$-LipL32**

The *E. coli* expression vector named pAE (Ramos et al. 2004) was used for cloning the LipL32 coding sequence, resulting in the plasmid named pAE/*lipL32*. This plasmid was used to transform *E. coli* BL21(DE3) pLysS, which was cultivated in liquid medium containing ampicillin. When the absorbance at 600 nm reached 0.8, isopropyl-1-thio-ß-D-galactoside (IPTG) was added to a final concentration of 1 mM and the cells were harvested by centrifugation 3 h later. After centrifugation at 10.000 × *g* for 5 minutes at 4°C, the cells were lysed by sonication, centrifuged once more and the supernatant submitted to protein purification by affinity chromatography in a nickel charged Sepharose column using the ÄKTAPrime chromatography system (Amersham Biosciences, USA). Fractions of the purified proteins were analyzed by 15% SDS-PAGE and Western blot (WB) using the 1D9 monoclonal antibody - MAb (Lüdtke et al. 2003). Fractions containing rLipL32 were dialyzed against PBS and glycine 0.1%, pH 8.0, for approximately 16 h at 4°C. Protein in the final preparation was quantified by the Bradford method (Bradford 1976).

**Preparation of rBCG and Western blot analysis**

The pUS973/*lipL32*, pUS974/*lipL32* and pUS977/*lipL32* vectors were introduced into *Mycobacterium bovis* BCG Pasteur strain by electroporation as previously described (Bastos et al. 2002). BCG transformants were grown for 15 days in Middlebrook 7H9 medium (Difco) containing 25 µg/ml of kanamycin. A volume of 3 ml was centrifuged, the pellet resuspended in 0.5 ml of 50 mM Tris, pH 7.5, and the cells lysed using a Ribolyser (Hybaid), three times (40 s) at speed 4. The lysate was centrifuged and the supernatant recovered. A volume of 50 µl of 2× loading buffer (50 mM Tris–HCl pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue) was added to 50 µl of the supernatant, the suspension heated to 100°C for 10 min and a volume of 10 µl was submitted to SDS-PAGE.

For Western blot analysis a total of 5 µg of rLipL32 or 50 µg of rBCG cell lysates and negative controls was separated in 15% SDS-PAGE resolving gels, and electrotransferred to nitrocellulose Hybond-C Super membrane (Amersham Biosciences). After blocking with 5% nonfat dry milk, the membranes were incubated for 1 h at 37°C with 1D9 MAb or pooled human sera at 1:100 dilution in PBS from convalescent patients diagnosed with leptospirosis (MAT titer of 25.000). After three washes of 20 min each with PBS containing 0.1% Tween 20 (PBS-T), the membranes were incubated for 1 h at room temperature with a secondary antibody conjugated with goat anti-mouse IgG peroxidase conjugate (Sigma) or goat anti-human IgG peroxidase conjugate, diluted in PBS-T and detected with ECL™ Western blot detection reagents (Amersham Biosciences, USA).

**Experimental animals**

BALB/c female mice were obtained from the University of São Paulo (ICB-USP) animal house. Experimental animals were housed at the animal facility of the Biotechnology Center of the Federal University of Pelotas (UFPel). The animals were maintained in accordance with the guidelines of the Ethics Committee in Animal Experimentation of the UFPel throughout the experimental period.

**Inoculation of mice and humoral immune response**

For evaluating the humoral immune response induced by the different vaccine preparations, mice aging from 5 to 6 weeks were allocated into 8 groups containing 5 animals each and inoculated twice, at day zero and 21, as described in table 2. Sera

from each group was collected from the retro-orbital plexus at days 0, 21, 42, 63, 84, 105, 126, 147 and 168 to monitor antibody responses, determined by ELISA against rLipL32. ELISA plates (Nunc Polysorp, Nalge Nunc International, USA) were coated overnight with 500 ng of rLipL32 per well, diluted in carbonate-bicarbonate buffer pH 9.6, washed three times and sera diluted 1:50 in PBS-T were added. After incubation for 1 h at 37°C, followed by three washes with PBS-T, peroxidase conjugated rabbit anti-mouse immunoglobulins (Sigma, USA) was added and the reaction visualized with o-phenylenediamine dihydrochloride (Sigma) and hydrogen peroxide. $OD_{450}$ were determined in a Multiskan MCC/340 (Titertek Instruments, USA) ELISA reader 15 min later. To avoid plate differences, the actual absorbances were transformed into seroconversions dividing them by that of day 0 of the serum from the same animal.

For Western blot analysis pool of sera from days 0 and 168 post inoculation of each experimental group were used. Cultures of *Leptospira interrogans* L1-130 in log phase were harvested, washed in PBS, resuspended in SDS-PAGE sample buffer and boiled for 10 min prior to separation by SDS-PAGE and electrotransference onto PVDF membrane (Amersham Biosciences, USA). Following blocking in 5% nonfat dry milk, the pool of sera diluted 1:25 was incubated at room temperature for 1 h. After five PBS-T washes, the membrane was incubated at room temperature for 1 h with 1:2.000 goat IgG anti-mouse Igs peroxidase conjugate. The reaction was revealed with 3-4-chloronaphtol after five PBS-T washes. Benchmarker™ Pre-stained Protein Ladder (Invitrogen, USA) was used as molecular mass standard.

**Indirect immunofluorescence**

Microscope slides (ICN Biomedicals, Inc) were coated with a 0.01% Poly L-Lysine solution (Sigma, USA) and dried for 1 h at room temperature. A 7-day culture of *L. interrogans* L1-130 was washed once in PBS, suspended in PBS to a density of $10^8$ cells/ml. A volume of approximately 1 ml was placed on the slide and incubated for 2 hours at 30°C. The slides were washed twice with leptospiral culture medium (LCM) and coated with pool of sera diluted 1:10 in LCM. The following groups were used in this experiment: (i) pTARGET/*lipL32*, (ii) Recombinant LipL32, (iii) rBCG (pUS9773/*lipL32*), (iv) rBCG (pUS9774/*lipL32*), (v) rBCG (pUS9777*lipL32*), (vi) MAb against LipL32 (1D9), (vii) MAb against S*almonella* OMP, (viii) rabbit anti-mouse FITC conjugate applied to slides without primary antibody, (ix) GroEL antiserum, and

(x) control mouse serum used as primary antibody. After incubating for 1 h at 30°C, the slides were washed twice with LCM and a 1:100 dilution of rabbit anti-mouse FITC conjugate was added and incubated for 1 h in a dark humid chamber at 30°C. After washing with LCM a drop of mounting medium was added and a cover slip was sealed with acrylic. Staining was visualized by fluorescence microscopy (Olympus) with excitation wavelength of 450 nm.

**In vitro *growth inhibition***

Leptospiral *in vitro* growth inhibition was performed as previously described (Tabata et al. 2002), with modifications. Briefly, a 7-day culture of *L. interrogans* L1-130 and *L. biflexa* patoc Patoc were grown to 2 x $10^8$ cells per ml and diluted in tubes with fresh leptospiral culture medium at final concentration of approximately 2 x $10^7$ cells per ml. A volume of 0.5 ml was incubated with twofold dilutions of heat-inactivated ascite fluid of anti-LipL32 MAb, with dilutions ranging from 1:5 to 1:320. Experiments were performed in triplicate for all dilutions tested. For test control, three tubes were added with heat-inactivated ascite fluid of an unrelated MAb, three tubes were added with heat-inactivated normal mouse serum (NMS) with 1:5, 1:10 and 1:20 final dilution and three tubes without any addition. All experiments were performed under sterile conditions; ascite MAbs and NMS were passed through a 0.22 µm membrane (Millipore). The cell growth was observed every other day by dark field microscopy to verify cell viability, including movement, morphology and agglutination. After 7 days the cultures were counted in a Petroff-Hausser chamber to determinate bacterial population. Growth inhibition was estimated by comparing number of live cells in the presence of MAbs and number of live cells in the presence of NMS.

**Statistical analyses**

Results of mice serological assays were compared using analysis of variance (ANOVA) and t-test (NCSS 2001©). Differences were considered significant at $P \leq 0.05$.

# RESULTS

## DNA Vaccine

The *lip*L32 gene was amplified and cloned into pTarget plasmid vector. A recombinant clone was selected and the presence and integrity of the insert was confirmed by *Bam*HI restriction digestion and DNA sequencing. A large scale plasmid DNA preparation was carried out and the DNA concentration was determined to be at 1 mg/ml. This plasmid preparation was subsequently used for mice immunization.

## Expression of the rLipL32

*E. coli* BL21 (DE3) pLysS transformed with the expression plasmid pAE/*lipL32* expressed a soluble recombinant protein of the expected size (30 kDa). Purification of rLipL32 from *E. coli* by affinity chromatography was highly efficient, resulting in approximately 40 mg per liter of medium. A single band was observed when the protein was submitted to SDS-PAGE (figure 1a).

Expression of LipL32 in BCG was evaluated by WB using the 1D9 MAb (figure 1b). This MAb recognized a protein of approximately 30 kDa in rBCG cell lysates and *L. interrogans* extracts. In contrast, no band was detected in wild type BCG extracts, demonstrating the specificity of this antibody. Based on band intensities, the level of expression of rLipL32 shown by recombinant BCG grown *in vitro* was considered to be similar for vectors pUS974/*lipL32* and pUS977/*lipL32*, but lower for pUS973/*lipL32* (figure 1b). WB with pooled sera from patients diagnosed with leptospirosis and crude extracts of rBCG strains expressing LipL32 or the purified rLipL32 revealed a positive reaction (data not shown).

## Humoral immune response

Seroconversions of BALB/c mice inoculated with the different vaccine preparations varied. The groups vaccinated with DNA vaccine (pTarget/*lipL32*), rBCG (pUS974/*lipL32* and pUS977/*lipL32*) and rLipL32 showed a seroconversion statistically different ($p < 0.05$) from the other groups (figure 2). The seroconversion of mice inoculated with rBCG/pUS974/*lipL32* or rBCG/pUS977/*lipL32* was still rising at the end of the experiment. The control groups inoculated with saline, pTarget and wild type BCG, as expected, did not show any seroconversion, however rBCG (pUS973/*lipL32*) also failed to stimulate a humoral immune response in mice.

Western blot analysis showed that sera from mice inoculated with DNA vaccine (pTarget/*lipL32*), rBCG (pUS974/*lipL32 and* pUS977/*lipL32*) and rLipL32 recognized native LipL32 present in *L. interrogans* extract (figure 3). No immuno-reactive bands were detected by WB using sera from the control animals and rBCG transformed with pUS973/*lipL32*, demonstrating that the humoral immune response induced by the different vaccine preparations was specific (data not shown).

**Indirect Immunofluorescence**

An indirect immunofluorescence assay revealed that antibodies induced by rBCG, DNA vaccine and rLipL32 were able to bind to LipL32 on the surface of the bacterium, suggesting that the antigen was properly presented to the immune system. Sera from groups of mice that showed seroconversion by ELISA, also recognized the LipL32 with intense fluorescence on intact bacterial cells (figure 4). When pooled serum from control groups was used, no fluorescence was observed.

***In vitro* growth inhibition by anti-rLipL32 MAb**

This approach was used to investigate the ability of a anti-rLipL32 MAb to inhibit the growth of *Leptospira in vitro.* The 1D9 anti-LipL32 MAb was bacteriostatic, since cell population remained constant over the incubation period in all dilutions tested. A slight morphology change and reduction in cell movement was observed after seven days of incubation with the 1D9 MAb. Bacterial growth was not affected either by a MAb against an unrelated antigen or NMS. No agglutination of bacterial cells was observed in any of the test tubes.

## DISCUSSION

Leptospirosis is an important zoonotic disease distributed worldwide (McBride et al. 2005). Immunization of livestock animals with bacterins is widely used, but the immune response is short-live, and animals require periodic boosters. Moreover, these bacterins provide no cross-protection against the different serogroups of pathogenic leptospires (Faine 1982; Yan et al. 2003; Martinez et al. 2004). Therefore the development of new strategies for the prevention of leptospirosis is necessary. Several studies indicate that the lipoprotein LipL32 is a promising vaccine antigen

candidate. It is the major outer membrane protein and is surface exposed (Cullen et al. 2005). Over 95% of patients with leptospirosis produce antibodies to LipL32 during infection (Flannery et al. 2001). In the present study, three different experimental immunization strategies against leptospirosis using LipL32 were evaluated: a subunit vaccine using purified recombinant LipL32; a live recombinant *Mycobacterium bovis* BCG expressing LipL32; and a DNA vaccine. All approaches were able to induce high levels of anti-LipL32 antibodies in BALB/c mice, which recognized the native LipL32 from *L. interrogans* by Western blot and indirect immunofluorescence. In addition, an anti-LipL32 MAb was shown to be able to inhibit growth of *Leptospira in vitro*, indicating potential protection by the LipL32 antigen.

Expression of LipL32 in BCG was achieved with the use of three different expression vectors. The plasmids pUS973 and pUS974 contain the mycobacterial *hsp60* gene promoter, which has been widely used to express heterologous antigens in BCG (Stover et al. 1991; Ohara and Yamada 2001; Dennehy and Williamson 2005). In addition, the plasmid pUS974 carries a signal sequence from the *M. tuberculosis* 19 kDa antigen (MT19). The MT19 signal sequence has been used to express heterologous proteins as lipoproteins on the mycobacterial surface (Ohara and Yamada 2001; Stover et al. 1993; Bastos et al. 2002). The pUS977 vector carries the $P_{AN}$ promoter of *M. paratuberculosis*, first characterized and isolated by (Murray et al. 1992). Since then, it has been used to express heterologous antigens from different pathogens (Ohara and Yamada 2001; Dennehy and Williamson 2005). Recombinant BCG transformed with pUS973/*lipL32* did not stimulated seroconversion against the recombinant antigen. The reason for this might be the *in vivo* instability of the plasmid construction. The strong promoter, present in pUS973 vector, has been shown to cause instability of the vector, both *in vitro* and *in vivo* (Medeiros et al. 2002). However, the pUS974/*lipL32* construct, which carries the same promoter, did not show the same instability, possibly due to the presence of the MT19 signal sequence.

In order to evaluate LipL32 as a naked DNA vaccine, we used the pTarget™ vector. The cytomegalovirus (CMV) promoter present in this vector has been shown to drive high levels of expression of recombinant antigens in eukaryotic cells, stimulating an effective immune response for over one year (Wolff et al. 1992). Mice inoculated with pTarget/*lipL32* presented a significant humoral immune response, which remained high for the duration of the experiment. A DNA vaccine containing

the *lipL32* (*hap-1*) coding sequence cloned into the pCDNA3.1 eukaryotic expression vector has been shown to induce protection against a lethal challenge with *L. interrogans* Canicola in the gerbil model (Branger et al. 2005). It remains to be evaluated whether the pTarget/*lipL32* construct is able to afford a similar level of protection.

Mice that received two doses of *E. coli* expressed rLipL32 presented a high seroconversion 42 days after the first inoculation. The seroconversion remained high until the end of the experiment, 160 days after the first inoculation. Previous attempts to induce protection in animal models using recombinant LipL32 failed in previous studies (Branger et al. 2001; Branger et al. 2005). It is conceivable that the mode of production or extraction of this recombinant protein, or its presentation to the immune system is unsuitable to induce a protective immune response, even though antibody production is obtained.

Comparing the immunization methods, all of them were able to develop specific humoral immune response against LipL32. Although the highest seroconversions were obtained with the purified recombinant protein, this might not be the most adequate form of presenting the antigen to the immune system. DNA immunization using the *lipL32* coding sequence has been shown to be protective against a lethal challenge (Branger et al. 2005). The use of BCG as a vaccine vector for the LipL32 antigen may constitute an efficient form of presenting this antigen to the immune system. As a live replicating bacterium, it keeps stimulating the immune response for a long period of time. Indeed, the groups of animals vaccinated with rBCG showed antibody titers that were still raising at the end of the experiment. In addition, recombinant BCG has been shown to induce a potent cellular immune response against foreign antigens (Bastos et al. 2002; Michelon et al. 2006). The protective immune response against leptospirosis is not fully characterized, however there are increasing evidences that the cellular immune response plays a major role (Naiman et al. 2001; Vernel-Pauillac and Merien 2006). Similarly to the DNA vaccine using *lipL32*, which was protective possibly to the capacity it has to induce not only humoral immune response, but primarily a cellular response, rBCG expressing LipL32 may be able to induce a protective immune response. The main advantages of using rBCG are the low cost of production and the long lived immune response.

One particularly important finding is that mice inoculated with DNA vaccine, rLipL32 and rBCG developed antibodies that recognized the native protein in the

intact membrane of *Leptospira interrogans*, by indirect immunofluorescence. This finding provides further evidence of LipL32 exposure on the surface of the bacterial cell.

The 1D9 MAb anti-LipL32, when incubated with live leptospiral cells, showed a bacteriostatic effect. This finding showed that the MAb was able to attach to a cell surface epitope and hamper the protein biological function in the outer membrane, indicating that an antibody immune response against LipL32 may contribute to protect an individual against infection by pathogenic leptospiras.

The present study described different strategies for inducing immune response against leptospirosis, including the use of LipL32 as a recombinant subunit vaccine, a DNA vaccine, and live recombinant BCG vaccine. To our knowledge this is the first report of expression of LipL32 in BCG. Humoral immune response, specific to the recombinant antigen, was demonstrated also to rBCG. Since the amino acid sequence of LipL32 is highly conserved among pathogenic leptospiras, immunoprotection induced by LipL32 may be broadly protective. We are currently investigating the efficacy of the different strategies of presenting the LipL32 to the immune system in inducing protection against challenge with pathogenic *L. interrogans,* using a hamster model.

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**TABLE 1.** Bacterial strains, plasmids, and primers used in this study

| Strain, plasmid or primer | Relevant information | Source or reference |
|---|---|---|
| ***Strain*** | | |
| *E. coli* DH5α | F⁻, *lacZ* △M15, *end*A1, *rec* A1, *sup* E44, *rel* A1 | Invitrogen, USA |
| *E. coli* BL21DE3 pLysS | [$F^-$ *ompT* $hsdS_B$ ($r_B^-$ $m_B^-$) *gal dcm* ▲(*srl-recA*)*306*::Tn*10*(TcR) (DE3) pLysS(CmR)] | Novagen, USA |
| *M. bovis BCG* Pasteur | Vaccine strain | FIOCRUZ-RJ |
| *Leptospira interrogans* | strain Fiocruz L1-130 was isolated from a patient during an outbreak of leptospirosis in Salvador, Brazil | (Ko et al. 1999) |
| ***Plasmids*** | | |
| pTarget | Mammalian Expression Vector , $Amp^r$, CMV promoter | Promega, USA |
| pAE | Cloning and Expression vector, $Amp^r$, T7 promoter | (Ramos et al. 2004) |
| pUS973 | *E. coli-mycobacteria* shuttle vector, $Kan^r$, oriM, promoter *hsp60* from *Mycobacterium tuberculosis* | (Medeiros et al. 2002) |
| pUS974 | *E. coli-mycobacteria* shuttle vector, $Kan^r$, oriM, *hsp60* promoter and signal sequence of *Mycobacterium tuberculosis* antigen 19 (MT19) | (Medeiros et al. 2002) |
| pUS977 | *E. coli-mycobacteria* shuttle vector, $Kan^r$, oriM, promoter $P_{AN}$ from *Mycobacterium paratuberculosis* | (Medeiros et al. 2002) |
| ***Primers*** | | |
| LipPTF | 5' ATGGGTGGTCTGCCAAGCCTAAAAAGCTC 3' | This work |
| LipPTR | 5'TTACTTAGTCGCGTCAGAAGCAGC3' | This work |
| LippAEF | 5'ccg**CTCGAG**GGTGGTCTGCCAAGCCT3' | This work |
| LippAER | 5'g**GAATTC**TTACTTAGTCGCGTCAGAAGC3' | This work |
| LipBCGF | 5'ta**TCTAGA**GGGTGGTCTGCCAAG3' | This work |
| LipBCGR | 5'cgg**AAGCTT**TTACTTAGTCGCG3' | This work |

In primer sequences, lowercase letters denote nucleotides added or modified to facilitate incorporation of restriction sites marked in bold.

**TABLE 2.** Groups of mice and vaccine preparations used in the experiment.

| Group | Immunogen | Dose | Route |
|---|---|---|---|
| Group A | pTARGET (control) | 100 μg of DNA | IM |
| Group B | pTARGET/*lipL32* | 100 μg of DNA | IM |
| Group C | Aluminum hydroxide | 15 % aluminum hydroxide | IM |
| Group D | Recombinant LipL32 | 100 μg of rLipL32 + 15 % aluminum hydroxide | IM |
| Group E | BCG (control) | $10^6$ CFU of BCG | IP |
| Group F | rBCG (pUS9773/*lipL32*) | $10^6$ CFU of BCG | IP |
| Group G | rBCG (pUS974/*lipL32*) | $10^6$ CFU of BCG | IP |
| Group H | rBCG (pUS977/*lipL32*) | $10^6$ CFU of BCG | IP |

IP: intraperitoneal injection; IM: intramuscular; CFU: colony-forming units.

## FIGURE CAPTIONS

**Figure 1**. (A) SDS-PAGE of purified rLipL32. Line 1, Protein Ladder (Invitrogen); lines 2-4, purified rLipL32 fractions. (B). Western blot with MAb 1D9 demonstrating LipL32 expression in BCG. Lane 1, rBCG transformed with pUS973/*lipL32*; lane 2, rBCG transformed with pUS974/*lipL32*; lane 3, rBCG transformed with pUS977/*lipL32*; lane 4, BCG (control).

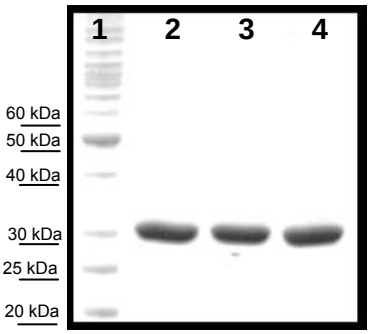
**Figure 2.** Mean seroconversion of anti-LipL32 systemic antibodies determined by ELISA from mice inoculated with different vaccine preparations. Mice were inoculated at days 0 and 21 of the experiment. **A**: evaluation of the immune response elicited by the DNA vaccine pTarget/*lipL32* and the rBCG constructs. **B**: evaluation of the immune response elicited by the subunit vaccine (purified rLipL32).

**Figure 3.** Western blot analysis of pooled sera from mice inoculated with different vaccines against crude extract of *L. interrogans*. 1, Benchmarker pre-stained protein ladder (Invitrogen); 2, pTarget/*lipL32* - day 0; 3, pTarget/*lipL32* - day 168 ; 4, rBCG (pUS974/*lipL32*) - day 0; 5, rBCG (pUS974/*lipL32*) - day 168; 6, rBCG (pUS977/*lipL32*) - day 0; 7, rBCG (pUS977/*lipL32*) - at day 168; 8, rLipL32 - day 0; 9, rLipL32 - day 168; 10, MAb 1D9.

**Figure 4.** Indirect Immunofluorescence with intact *L. interrogans*. Panel A. Pooled sera from animals vaccinated with the DNA vaccine (pTarget/*lipL32*); Panel B. rBCG (pUS974/*lipL32*); Panel C. rBCG (pUS977/*lipL32*); Panel D. rLipL32; Panel E. Pooled sera from the saline group.

**FIGURE 1**

A


1
2
3
4
60 kDa
50 kDa
40 kDa
30 kDa
25 kDa
20 kDa

B

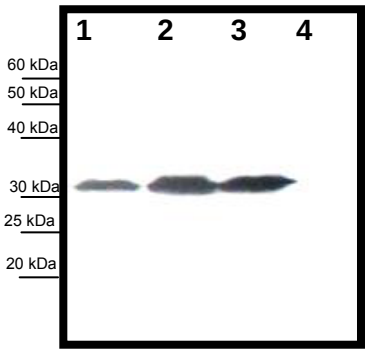
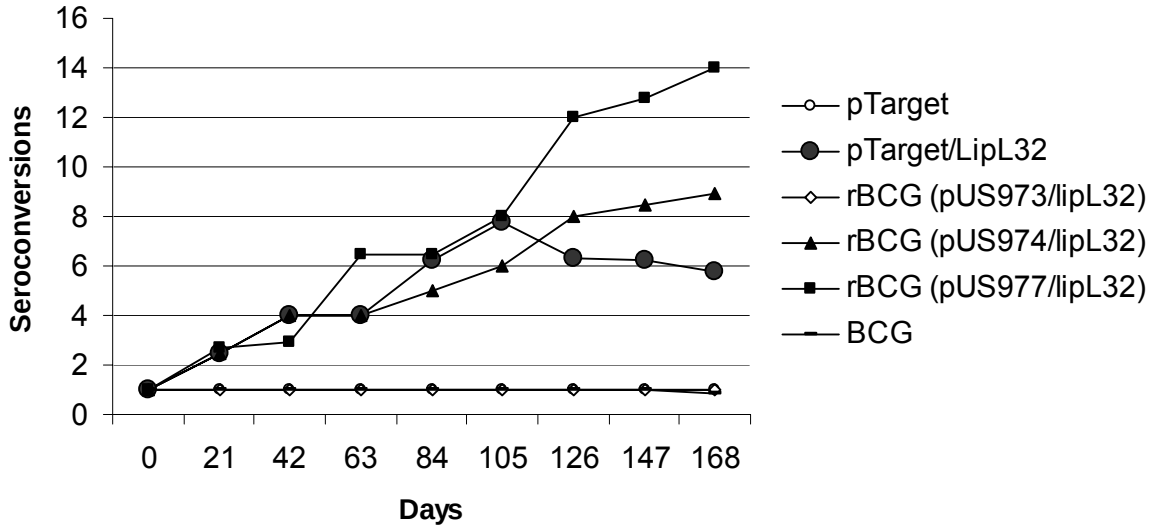

1
2
3
4
60 kDa
50 kDa
40 kDa
30 kDa
25 kDa
20 kDa

**FIGURE 2**

**A**


16
14
12
10
8
6
4
2
0
Seroconversions
0
21
42
63
84
105
126
147
168
Days
pTarget
pTarget/LipL32
rBCG (pUS973/lipL32)
rBCG (pUS974/lipL32)
rBCG (pUS977/lipL32)
BCG

**B**

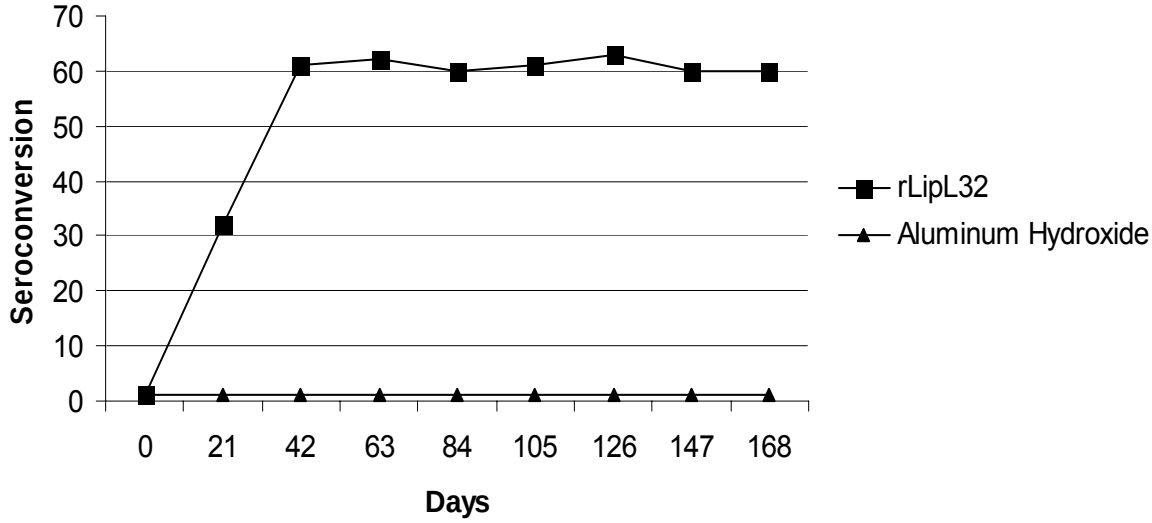

70
60
50
40
30
20
10
0
Seroconversion
0
21
42
63
84
105
126
147
168
Days
rLipL32
Aluminum Hydroxide

**FIGURE 3**

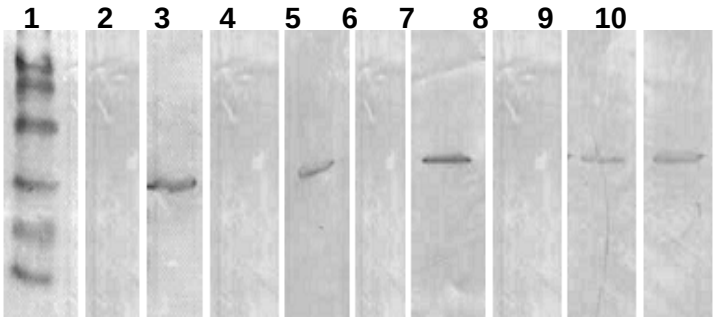
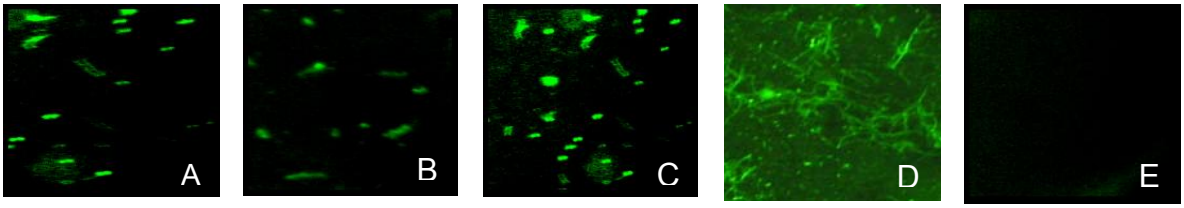


**FIGURE 4**



# 4 ARTIGO 3

## RECOMBINANT *Mycobacterium bovis* BCG EXPRESSING THE LipL32 ANTIGEN OF *Leptospira interrogans* PROTECTS HAMSTERS FROM CHALLENGE

(Artigo que será submetido à Vaccine)

# RECOMBINANT *Mycobacterium bovis* BCG EXPRESSING THE LipL32 ANTIGEN OF *Leptospira interrogans* PROTECTS HAMSTERS FROM CHALLENGE

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## ABSTRACT

The bacillus Calmette-Guerin (BCG), a live attenuated *Mycobacterium bovis* strain is considered a promising candidate as a vector system for delivery of foreign antigens to the immune system. The gene coding for the *Leptospira interrogans* external membrane protein LipL32, a highly immunogenic antigen found in all pathogenic leptospira, was cloned into several mycobacterial vectors for expression in BCG. Hamsters immunized with recombinant BCG (rBCG) expressing LipL32 were protected against mortality ($P \leq 0.05$) upon challenge with a lethal inoculum of *L. interrogans* serovar Copenhageni. Autopsy examination did not reveal macroscopic or histological evidence of disease in rBCG immunized hamsters that survived lethal challenge. The data presented here further enhance the status of LipL32 as a promising candidate antigen for use in the control of leptospirosis, when presented to the immune system by an appropriate delivering system.



**Key Words:** Leptospirosis, recombinant BCG, LipL32


**Running headline:** rBCG expressing LipL32 protects against leptospirosis

## INTRODUCTION

Leptospirosis is an infectious disease caused by spirochetes belonging to the genus *Leptospira*. It is considered the most widespread zoonosis in the world [1]. With large outbreaks occurring during the last decade in Nicaragua [2], Brazil [3], and India [4], leptospirosis is now recognized as an important re-emerging infectious disease [5].

Development of an effective vaccine against leptospirosis with cross immunoprotection against different serovars remains a challenge. Efforts to develop recombinant leptospiral vaccines have therefore focused on outer membrane proteins [6-12]. LipL32, also called haemolysis-associated protein 1 (Hap1), is amongst the promising vaccine candidates. It is the most abundant of the outer membrane proteins and its expression has been demonstrated during both, *in vitro* cultivation and host infection [8]. It is exposed on the surface of the cell [7, 13] and plays an important role in leptospiral infection [14, 15]. Over 95% of patients with leptospirosis produce antibodies to LipL32 during infection [16]. Moreover, the nucleotide sequence coding for LipL32 is conserved among pathogenic leptospira, whereas it is absent in nonpathogenic leptospira [8]. Immunization of gerbils with an adenovirus construct encoding LipL32 or a DNA vaccine construct containing the *lipL32* gene has been found to provide partial protection from lethal challenge [6, 17].

*Mycobacterium bovis* BCG is a live attenuated vaccine used to protect against tuberculosis [18]. BCG offers unique advantages as a vaccine: it is unaffected by maternal antibodies and therefore it can be given at any time after birth, it is usually given as a single dose eliciting a long-lasting immunity, it is stable and safe, it can be administrated orally, and it is inexpensive to produce when compared with other live vaccines [19]. The extraordinary adjuvant properties of mycobacteria make them an attractive vector for the development of recombinant vaccines [20]. It has been shown that recombinant BCG (rBCG) elicits both cellular and humoral immune response against heterologous antigens [21]. In fact, rBCG has already been shown to provide protection against *Borrelia burgdorferi* [22], *Leishmania* [23], *Streptococcus pneumoniae* [24], malaria [25], *Schistosoma mansoni* [26] and enhanced protection against *M. tuberculosis* [27, 28].

In our previous study, rBCG expressing LipL32 was demonstrated to be immunogenic in mice [29]. In this study, we report the cloning and expression of

LipL32 in *M. bovis* BCG. Hamsters inoculated with the rBCG constructs developed a specific immune response and protection against challenge with a virulent strain of *Leptospira*. These results indicate that *M. bovis* BCG is an adequate vector to express and present LipL32 to the immune system, and may therefore be used to further study the induction of protective immunity against human and animal leptospirosis.

## MATERIALS AND METHODS

*Bacterial strains and growth conditions*

*Escherichia coli* strain TOP10 (Invitrogen, USA) was grown in Luria-Bertani medium at 37 ºC with the addition of the appropriate antibiotic (kanamycin 50 µg/ml). *M. bovis* BCG Pasteur was grown in Middlebrook 7H9 broth (Difco, USA) supplemented with 10% of oleic acid, albumin, dextrose complex (OADC – Difco), 0.2% glycerol and 0.05% Tween 80 (Sigma, USA), or 7H11 agar (Difco) containing 10% OADC and 0.2% glycerol, with or without kanamycin (25 µg/ml). *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was grown at 28ºC in Ellinghausen-McCullough-Johnson-Harris (EMJH) enriched liquid medium (Difco).

*Preparation of rBCG and Western blot analysis*

Four *E. coli*-mycobacteria shuttle vectors were used. They have the same backbone, differing only in the promoter used to drive the expression of the foreign gene: pUS973 (*hsp60* promoter), pUS974 (*hsp60* promoter plus MT19 signal sequence), pUS977 ($P_{AN}$ promoter) and pUS2000 (*18kDa* gene promoter). The pUS973/*lipL32*, pUS974/*lipL32,* pUS977/*lipL32* and pUS2000/*lipL32* vectors were constructed and introduced into *M. bovis* BCG Pasteur strain by electroporation as previously described [29].

Expression of LipL32 in BCG was demonstrated using Western blot (WB). A volume of 10 ml of each recombinant mycobacterial culture was harvested at mid-log phase by centrifugation at 4000 × *g* for 15 min, and resuspended in 0.05 M Tris–HCl pH 7.4. The bacteria were then disrupted in a ribolyser (Hybaid) for 45 s, power 5. Sample buffer (2% SDS, 0.1 M DTT, 0.01% Bromophenol, 0.06 M Tris–HCl, 10% glycerol) was added to the extracts, which were boiled for 10 min. After boiling, the

extracts were centrifuged at 13,000 × *g* for 1 min, and the supernatant was recovered. A total of 50 µg of mycobacterial proteins was separated on a 15% SDS-PAGE resolving gel and electrotransferred to a PVDF membrane (GE Healthcare).

The membranes were blocked using 5% skimmed milk powder for 1 h at room temperature. The monoclonal antibody 1D9, specific against the LipL32 [13] was used to detect the recombinant antigen. Incubation with the primary antibody was performed at room temperature for 1 h. After washing with PBS-T (phosphate buffer saline pH 7.6 with 0.05% Tween 20), for 30 min, the membrane was incubated for 1 h with goat anti-mouse IgG peroxidase conjugate (Sigma) and detected with ECL™ Western blot detection reagents (GE Healthcare).

*Animals and $LD_{50}$ determination*

Male and female Golden Syrian hamsters used in all experiments were obtained from the Central Animal Facility of the Federal University of Pelotas (UFPel). The experimental animals were housed at the animal facility of the Biotechnology Centre – UFPel, and maintained in accordance with the guidelines of the Ethics Committee in Animal Experimentation of the UFPel throughout the experimental period.

For determination of the $LD_{50}$, groups of four twenty-week-old hamsters, matched by sex for each dilution, were infected intraperitoneally with 10 fold serial dilution ($10^4$ to $10^1$) in a final volume of 1 ml of PBS. Animals were monitored daily for clinical outcome until 28 days post-infection. The $LD_{50}$ was calculated by the method of Reed and Muench [30]. Negative control animals were injected with the same volume of EMJH media alone. This project and all animal experiments were approved by the Committee for Animal Care and Use (UFPel).

*Inoculation of hamsters with rBCG*

Wild type BCG (wtBCG) and rBCG (BCG/*lipL32*) were grown in 7H9 medium enriched with 10% OADC without or with kanamycin, respectively. The bacterial concentration was estimated by spectrophotometry at $OD_{600}$, and inoculated into hamsters. In the first experiment female hamsters were randomly allocated in six treatments, and inoculated with wtBCG (control) (A), rBCG (pUS973/*lipL32*) (B), rBCG (pUS974/*lipL32*) (C), rBCG (pUS977/*lipL32*) (D), rBCG (pUS2000/*lipL32*) (E) and killed whole-leptospires (F). All animals were inoculated intraperitoneally (i.p), in

days 0 and 21 of the experiment, with approximately $10^6$ CFU of wtBCG or rBCG in 100 µl of sterile PBS-T (Table 1). A positive control group was immunized with killed whole-leptospires. Washed pellets of cultures *L. interrogans* strain Fiocruz L1-130 were heat-inactivated 56°C for 20 min, resuspended in PBS and stored at -20°C until use. Hamsters were immunized with a dose of $10^9$ inactivated leptospires on day 0 and day 21. Prior to inoculation, expression of LipL32 by the rBCG constructs was confirmed by WB. Blood samples were collected from the retro-orbital plexus every 21 days with sera stored at −20 °C, for using in ELISA and WB. Seventy days after the first dose all animals were challenged with a lethal inoculum of *L. interrogans* serovar Copenhageni.

*Evaluation of the humoral immune response*

Antibody responses were monitored by indirect ELISA and WB using purified rLipL32 or whole cell *L. interrogans* extract. Polystyrene plates were coated overnight with 500 ng of rLipL32 per well, diluted in carbonate–bicarbonate buffer pH 9.6, washed three times and sera diluted 1:50 in PBS-T were added. After incubation for 1 h at 37 ºC, followed by three washes with PBS-T, goat anti-hamster IgG peroxidase conjugate (Serotec, USA) was added and the reaction visualized with *o*-phenylenediamine dihydrochloride (Sigma) and hydrogen peroxide. Absorbance was determined at 450 nm in Multiskan MCC/340 (Titertek Instruments, USA) ELISA reader. Mean values were calculated from serum samples assayed in duplicate. To avoid plate differences the actual absorbances were transformed in seroconversions dividing the absorbance of each serum by that of the preimmune serum.

For WB a total of 5 µg of rLipL32 or 50 µg of crude *L. interrogans* extract was separated on a 15% SDS-PAGE resolving gels, and electrotransferred to a PVDF membrane (GE Healthcare). After blocking, membranes were incubated for 1 h at 37 ºC with sera from experimental animals diluted 1:10 in PBS-T. After washing with PBS-T for 30 min, membranes were incubated for 1 h with goat anti-hamster IgG peroxidase conjugate (Serotec, USA), diluted 1:2000. The reaction was developed with 4-chloro-1-naphthol (Sigma) after five washes with PBS-T. Benchmarker™ Prestained Protein Ladder (Invitrogen) was used as molecular weight marker.

*Hamster challenge experiments*

Tree challenge experiments were conducted with immunized hamsters. In the first experiment, female hamsters in groups of eight were immunized two times i.p. at 21 days intervals as described above. Seventy days after the first dose all animals were challenged i.p. with $10^2$ virulent *L. interrogans* strain Fiocruz L1-130. In the second and third experiments only pUS977/*lipL32,* pUS2000/*lipL32* and controls were used. The second experiment was carried out with six animals per group, whereas the third one had 20 animals per group.

Hamsters were monitored daily for clinical signs of leptospirosis and euthanized when clinical signs of terminal disease appeared. Surviving hamsters on day 28 post-challenge were euthanized. Kidney and lung tissues were harvested for culture and histopathology studies. Sterilizing immunity was determined based on culture isolation of leptospires and identification of leptospirosis-associated pathology in surviving hamsters. From each organ, 1–2 g of tissue was aseptically removed, transferred to 10 ml of phosphate-buffered saline (pH 7.2), and homogenized. One hundred-fold dilutions of the homogenate were used to inoculate EMJH medium. Dark-field microscopy was performed during an 8 week incubation period to identify positive cultures. For histopathological studies, lungs and kidney tissues were fixed with 10% formalin (pH 7.0), and then embedded in paraffin. Sections of 5- to 6-µm were stained with hematoxylin and eosin for evidence of interstitial nephritis or pulmonary haemorrhage.

*Statistical analysis*

The Students t-test was used to determine significant differences in serological assays. The Fisher Exact test and log-rank sum test were used to determine significant differences for mortality and survival, respectively, among the groups immunized with rBCG and the negative control group. Differences were considered significant at $P \leq 0.05$.

## RESULTS

*Expression of the rLipL32 in BCG*

Expression of LipL32 in BCG was evaluated by WB using the 1D9 MAb. This MAb recognized a protein of approximately 30 kDa in rBCG cell lysates and *L. interrogans* strain Fiocruz L1-130 extracts. In contrast, no band was detected in wild type BCG extract, demonstrating the expression of LipL32 in BCG. Based on band intensities, the level of expression of rLipL32 shown by recombinant BCG grown *in vitro* was considered to be similar for all vectors used (Figure 1).

*Humoral immune response elicited by rBCG in hamsters*

Hamsters were vaccinated with wtBCG (control), rBCG (pUS973/*lipL32)*, rBCG (pUS974/*lipL32)*, rBCG (pUS977/*lipL32)* and rBCG (pUS2000/*lipL32)*, on days 0 and 21 of the experiment. Blood samples were collected every 21 days and the humoral immune response against rLipL32 was evaluated by indirect ELISA (Figure 2) and WB. Forty-two days after administration of the first dose, significant seroconversion ($P \leq 0.05$) was detected in hamsters vaccinated with rBCG (pUS974/*lipL32)*, rBCG (pUS977/*lipL32)* and rBCG (pUS2000/*lipL32)*, but not with wtBCG (control) and rBCG (pUS973/*lipL32)*. The rBCG (pUS2000/*lipL32)* construct induced seroconversion of 11 times above pre-immunization levels 63 days post-initial vaccination, rBCG (pUS977/*lipL32)* induced a seroconversion of only six times at day 63 and rBCG (pUS974/*lipL32)* induced a lower seroconversion of only four times at day 63. The control groups inoculated with wild type BCG, as expected, did not show any seroconversion. However rBCG (pUS973/*lipL32*) failed to stimulate a humoral immune response in hamster. A similar result was found when this construct was used to immunize mice [29].

Immunoblot analysis showed that sera from hamsters inoculated with rBCG (pUS974/*lipL32,* pUS977/*lipL32* and pUS2000/*lipL32*) recognized native LipL32 present in *L. interrogans* strain Fiocruz L1-130 extract (data not show). No immuno-reactive bands were detected by WB using sera from the control animals and rBCG transformed with pUS973/*lipL32*, demonstrating that the humoral immune response induced by the different vaccine preparations was specific.

*Determination of $LD_{50}$ for i.p. challenge of hamsters*

Since lethality of some leptospiral strains can be variable in older hamsters and we planned to use prolonged immunization schedules, it was necessary to standardize the animal model and to determine the $LD_{50}$ at the time of challenge. We found that 12-week-old hamsters were highly susceptible to lethal infection after i.p. inoculation with low-passage *L. interrogans* strain Fiocruz L1-130. The survival at 28 days after challenge was 50% or less for all doses tested in male and 25% or less for all doses tested in female indicating that the $LD_{50}$ for i.p. challenge of hamsters with *L. interrogans* strain Fiocruz L1-130 is approximately 20 bacteria for female and less than 5 bacteria for male (Table 2). The time to death after challenge was between 9 and 13 days (Table 2).

*Immunoprotection of hamsters immunized with rBCG/lipL32*

Female hamsters were challenged with $10^2$ leptospires (5 × $LD_{50}$ for female hamsters) 70 days after the first BCG immunization, and mortality rates were recorded 28 days after challenge. Three experiments were performed. The results of a statistical analysis of mortality (Fisher test) are show in Table 3. In the first experiment, mortality rates for animals vaccinated with rBCG (pUS973/*lipL32*) or rBCG (pUS974/*lipL32*) and animals vaccinated with wtBCG were not significantly different. Based on this result, and the fact that these vaccine preparations elicited a lower humoral immune response, they were excluded from subsequent experiments. The rBCG (pUS977/*lipL32*) and rBCG (pUS2000/*lipL32*) afforded statistically significant protection against lethal challenge in comparison to the wtBCG ($p \leq 0{,}038$) in the first experiment. Protection afforded by rBCG (pUS2000/*lipL32*) was statistically significant in all three experiments (Table 3).

The consistent lethal infection in the wtBCG control group demonstrated the reproducibility of the conditions used in all experiments. Any possible effect of immunization is demonstrated by the improved survival rate in the rBCG vaccinated groups. This consistence allows the analysis of the combined results of all protection experiments (Figure 3).

The survival rate of animals vaccinated with rBCG (pUS973/*lipL32*) or (pUS974/*lipL32*) (12.5%) was similar to the survival rate of the wtBCG control group (10%). However, the combined survival rate of animals vaccinated with rBCG (pUS977/*lipL32*) (32.4%) and (pUS2000/*lipL32*) (55.9%) was significantly higher ($p <$

0.05) than the control group. In addition, animals vaccinated with these two rBCG constructs that died during the course of the experiment, survived longer then the animals form the control group (figure 4).

*Necropsy and histopathological examination*

Recombinant BCG immunized hamsters that survived the lethal challenge, as well as animals vaccinated with the whole *Leptospira* preparation, did not show clinical evidence of infection during the 28 day follow-up period after challenge. Autopsy examination did not find macroscopic or histological evidence of disease in these animals, whereas severe pathological lesions were found in animals vaccinated with wtBCG which died during the course of the experiment (Figure 5). Isolation of leptospira from lung and kidney tissue of surviving animals was negative, indicating that the rBCG/lipL32 as well as the whole-cell vaccine conferred sterilizing immunity. In contrast, all tissue samples obtained from animals from the wtBCG control group were positive.

## DISCUSSION

Our previous work evaluated three experimental immunization strategies against leptospirosis using LipL32 as a model antigen. We showed that a subunit vaccine with purified recombinant LipL32, a live recombinant *M. bovis* BCG expressing LipL32, and a DNA vaccine were able to induce high levels of anti-LipL32 antibodies in BALB/c mice, which recognized the native LipL32 from *L. interrogans* by WB and indirect immunofluorescence. In addition, an anti-LipL32 MAb was shown to be able to inhibit growth of *Leptospira in vitro*, indicating potential protection by the LipL32 antigen [29].

The major objective of this study was to assess the potential of recombinant *M. bovis* BCG expressing LipL32 as a vaccine candidate against leptospirosis, using the standard hamster model. Previous studies had already demonstrated that LipL32 is able to induce protective immunity in the gerbil model of leptospirosis, when expressed in adenovirus [6] or as a naked DNA vaccine [17], but has no protective effect when administered as a recombinant subunit vaccine produced in *E. coli*. Recombinant BCG has the capacity of inducing a strong cellular as well as humoral immune response against foreign antigens [21]. It is conceivable that not only

humoral, but also cellular immune response plays an important role in the immunoprotection against leptospirosis. If that is the case, then BCG is a suitable expression system for *Leptospira* antigens and deserves further evaluation.

Expression of LipL32 in BCG was achieved with the use of four different expression vectors. The plasmids pUS973 and pUS974 contain the mycobacterial *hsp60* gene promoter, which has been widely used to express heterologous antigens in BCG [19-21]. In addition, pUS974 carries a signal sequence from the *M. tuberculosis* 19 kDa antigen (MT19). The MT19 signal sequence has been used to express heterologous proteins as lipoproteins on the mycobacterial surface [31-33]. The pUS977 vector carries the $P_{AN}$ promoter of *M. paratuberculosis*, first characterized and isolated by Murray et al. [34]. The pUS2000 vector contains the 18 kDa gene promoter from *M. leprae*, which has been shown to be up-regulated *in vivo* [35]. Despite the fact that the level of expression of the recombinant antigen was similar during *in vitro* growth, the immune response afforded by the different constructs was diverse. Vectors containing the *hsp60* gene promoter, namely pUS973 and pUS974, fail to induce a significant immune protection. The pUS973/*lipL32* also failed to induce an antibody response. The reason for that might be the strength of the promoter, which makes de vector unstable during in vivo growth, as previously demonstrated with similar constructs [36]. The superior stability of pUS977 and pUS2000, comparing to vectors containing the *hsp60* promoter, has been demonstrated [37, 38].

Seroconversion induced by rBCG (pUS2000/*lipL32*) was approximately 5 times higher than that induced by rBCG (pUS977/*lipL32*), which in turn was also approximately 5 times higher than the seroconversion induced by rBCG (pUS974/*lipL32*). It can be speculated that *in vivo* the yield of LipL32 driven by the *18kDa* promoter was greater than that produced by the $P_{AN}$ promoter. Challenge experiments showed that both rBCG (pUS977/*lipL32*) and rBCG (pUS2000/*lipL32*) elicited protective immunity in hamsters. The level of protection had a positive correlation with the humoral immune response, however, evaluation of the cellular immune response elicited by the rBCG constructs against the LipL32 antigen is needed for better understanding the nature of the protective response. It is intriguing the fact that recombinant LipL32 produced in *E. coli* was not able to induce a protective response despite eliciting a strong humoral immune response [6] (our unpublished observation). DNA vaccine, recombinant adenovirus and recombinant BCG are known to induce a strong cellular response. It remains to be elucidated

whether LipL32 induces a protective response when presented by these three different delivery systems because they are able to stimulate a cellular response, or because they are able to produce a properly folded protein, what *E. coli* fails to do. In contrast the *Leptospira* immunoglobulin-like protein LigA induces a protective immune response when used as a recombinant protein produced in *E. coli* [39-41] suggesting that protection is predominantly antibody mediated, though the participation of the cellular immune response can not be ruled out.

An important finding is that rBCG/*lipL32* was able to induce a sterilizing immunity. Animals immunized with rBCG (pUS977/*lipL32*) or rBCG (pUS2000/*lipL32*) that survived the lethal challenge with 5 × the $LD_{50}$ of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 not only showed no clinical or histopathological signs of disease, but also presented negative results in the attempt to isolate bacteria from lungs and kidneys. The C-terminal portion of LigA was able to induce protection when administered with complete Freund adjuvant, however surviving animals still harboured the bacteria in the kidneys [41].

The data presented here further enhance the status of LipL32 as a promising candidate antigen for use in the control of leptospirosis and show that the use of rBCG can be considered as a convenient means of eliciting a long lived immune response. We are currently investigating the use of a new *M. bovis* BCG expression system based on auxotrophic complementation as selectable marker, improving vector stability *in vivo* without the use of antibiotic resistance marker. In addition, we are evaluating BCG as a vaccine vector for other potentially protective leptospiral antigens. The combination of improved BCG expression system and expression of other antigens can result in an effective recombinant vaccine against leptospirosis for human and animal use.

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**TABLE 1.** Groups of hamster and vaccine preparations used in the experiment.

| Group | Immunogen | Dose | Route |
|---|---|---|---|
| Group A | wtBCG (control) | $10^6$ CFU of BCG | IP |
| Group B | rBCG (pUS973/*lipL32*) | $10^6$ CFU of BCG | IP |
| Group C | rBCG (pUS974/*lipL32*) | $10^6$ CFU of BCG | IP |
| Group D | rBCG (pUS977/*lipL32*) | $10^6$ CFU of BCG | IP |
| Group E | rBCG (pUS2000/*lipL32*) | $10^6$ CFU of BCG | IP |
| Group F | killed whole-leptospires | $10^9$ leptospires | IP |

IP: intraperitoneal injection; CFU: colony-forming units.

**TABLE 2.** Determination of $LD_{50}$ for *L. interrogans* strain Fiocruz L1-130 in the Golden Syrian hamster infection model.

| Leptospiras | Female | Death | Lethality | Male | Death | Lethality |
|---|---|---|---|---|---|---|
| 10000 | 4/4 | 9,9,10,10 | 100% | 4/4 | 9,9,10,10 | 100% |
| 1000 | 4/4 | 9,9,11,11 | 100% | 4/4 | 10,10,11,11 | 100% |
| 100 | 4/4 | 10,10,12,12 | 100% | 4/4 | 10,10,11,13 | 100% |
| 10 | 2/4 | 11,13 | 50% | 3/4 | 11,11,13 | 75% |

**TABLE 3.** Protective effect of immunization with rBCG/*lipL32* against lethal challenge in the hamster model

| Treatment group | Expt 1 | Expt 2 | Expt 3 | No. of surviving/total animals |
|---|---|---|---|---|
| rBCG (pUS973/*lipL32*) | 1/8 | - | - | 1/8 |
| rBCG (pUS974/*lipL32*) | 1/8 | - | - | 1/8 |
| rBCG (pUS977/*lipL32*) | 4/8* | 1/6 | 6/20 | 11/34* |
| rBCG (pUS2000/*lipL32*) | 4/8* | 3/6 | 12/20* | 19/34* |
| BCG (Control) | 0/8 | 0/6 | 4/20 | 4/34 |
| Killed whole leptospires | 8/8* | 6/6* | 20/20* | 34/34* |

* Protection against lethal challenge was statistically significant ($P < 0.05$).

## FIGURE LEGENDS

**FIGURE 1.** Western blot developed with MAb 1D9 demonstrating LipL32 expression in BCG. Lane 1, rBCG transformed with pUS973/*lipL32*; lane 2, rBCG transformed with pUS974/*lipL32*; lane 3, rBCG transformed with pUS977/*lipL32*; lane 4, rBCG transformed with pUS2000/*lipL32* and lane 5, wtBCG (control).

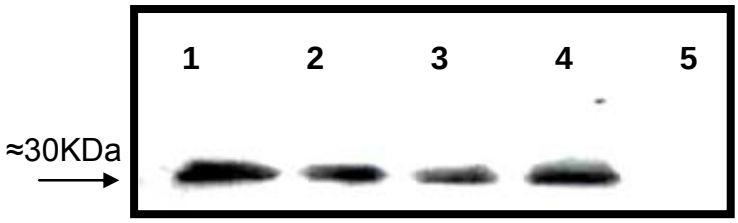
**FIGURE 2.** Seroconversion of total antibodies anti-LipL32 of hamsters inoculated with wtBCG, rBCG (pUS973/*lipL32)*, rBCG (pUS974/*lipL32)*, rBCG (pUS977/*lipL32)* or rBCG (pUS2000/*lipL32*), expressed in ELISA units. Recombinant LipL32 was used as antigen in the ELISA. Results are expressed as mean seroconversion, for pool serum samples. $^{*}p < 0.05$ in comparison to the control groups. (IP) Intraperitoneally immunized animals. (C) Intraperitoneally challenged. Each point corresponds to the pool of sera of the corresponding group of animals from the first experiment.

**FIGURE 3.** Percentage of survival of hamsters challenged with *L. interrogans* L1130. Asterisks denote a significant difference in survival rate when compared to the control group ($p < 0.05$). Data represent all experiments summarized.

**FIGURE 4.** Survival of hamsters challenged with *L. interrogans* L1130 after immunization with rBCG. Hamsters were immunized with wtBCG (control), rBCG (pUS977/*lipL32*) and rBCG (pUS2000/*lipL32*). The log-rank sum test was used to determine significant differences for survival, between the groups immunized with rBCG and the negative control group ($p \leq 0.05$). Data represent the combined results of three separate experiments.

**FIGURE 5.** Histopathological analysis of hamster tissues stained with hematoxylin and eosin. (A) and (C) Kidney from hamster vaccinated with rBCG/*lipL32* and challenged with $10^2$ *L. interrogans* cells. Note the normal renal architecture. (B) and (D) Kidney from hamster vaccinated with control wtBCG and challenged. Note pathologic changes in the kidney. The interstitium is infiltrated by small numbers of lymphocytes and plasma cells (long arrow). (E) Lung from hamster vaccinated with rBCG and challenged. Note the normal lung (F) Lung from hamster vaccinated with control wtBCG and challenged. Haemorrhage of the lung (long arrow).

*Vaccine. Seixas et al., 2007. Figura 1.*

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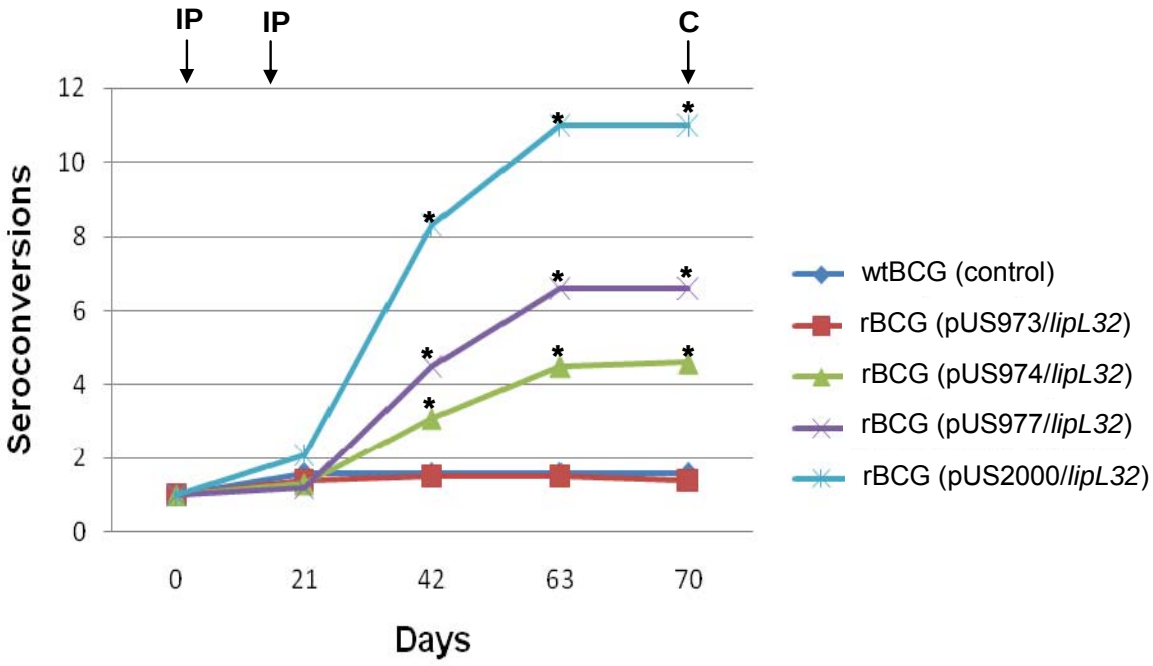

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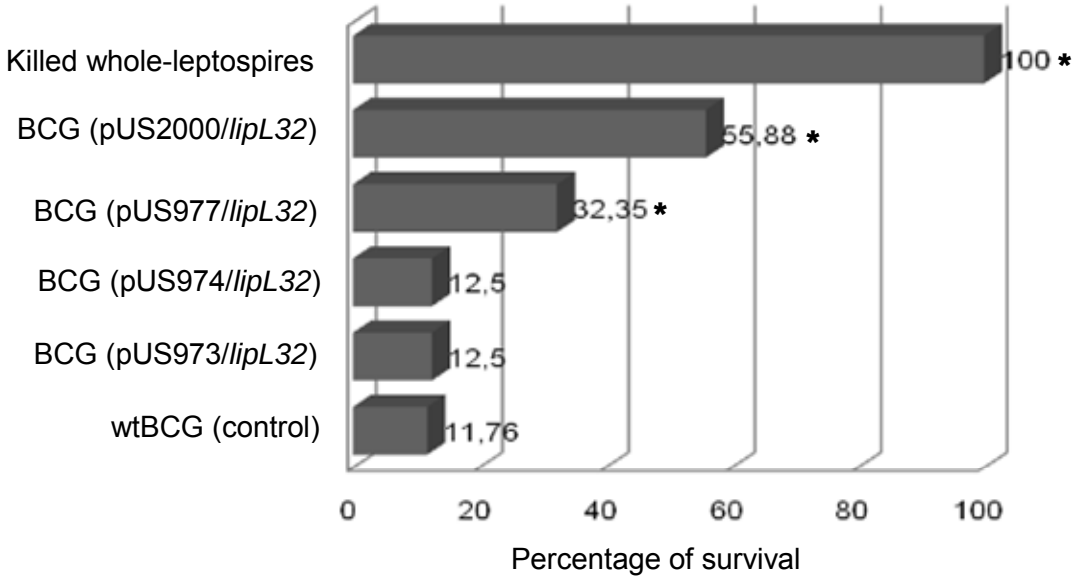

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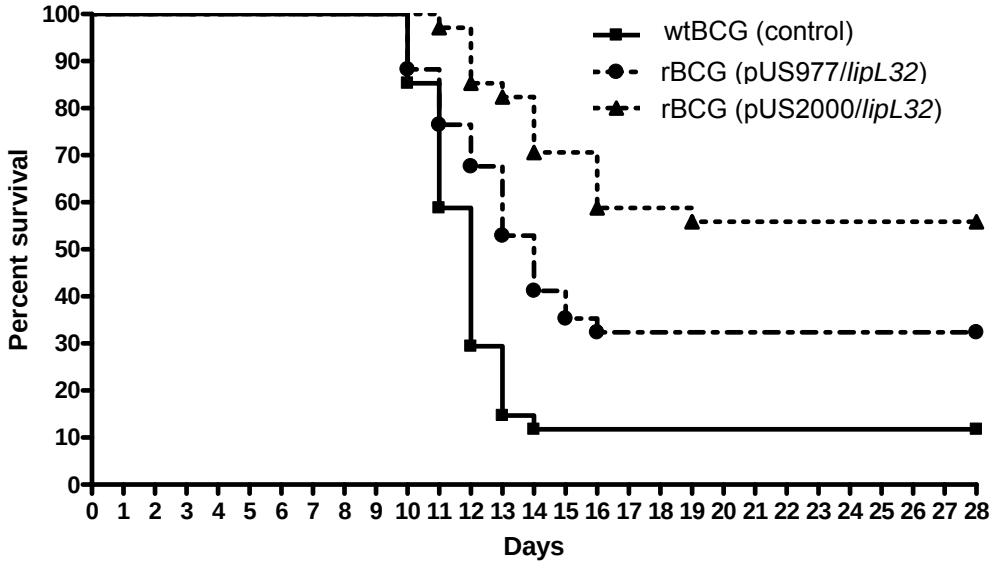

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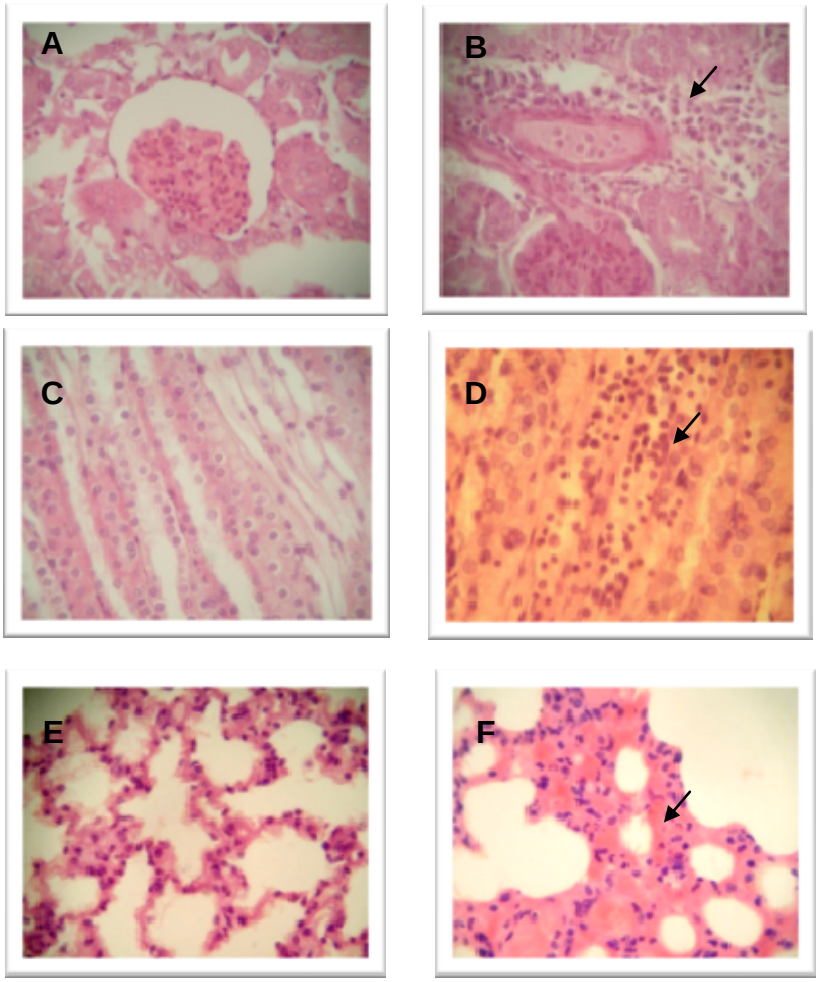


# 5 ARTIGO 4

## STABILITY EVALUATION OF AUXOTROPHIC *Mycobacterium bovis* BCG EXPRESSING LEPTOSPIRAL ANTIGENS IN HAMSTERS MODELS

(Artigo em processo de submissão ao periódico FEMS Microbiology Letters)

# STABILITY EVALUATION OF AUXOTROPHIC *Mycobacterium bovis* BCG EXPRESSING LEPTOSPIRAL ANTIGENS IN HAMSTERS MODELS


## ABSTRACT

Stability of the expression vector during *in vivo* growth is crucial for induction of a long lived immune response by recombinant *Mycobacterium bovis* BCG. We compared the expression of two *Leptospira interrogans* antigens, LipL32 and LigAni, in a conventional system and in a complementation vector system that used an auxotrophic strain of BCG (BCG ∆*leuD)*. Stability of the plasmids was evaluated during *in vivo,* after inoculation of the recombinante BCG in hamsters. The new system was highly stable as the selective pressure is maintained during *in vivo* growth, whereas the conventional system was unstable in the absence of selective pressure.


**Keywords:** BCG recombinant, auxotrophic complementation, foreign antigens

## INTRODUCTION

*Mycobacterium bovis* BCG is one of the attractive candidates for the development of live recombinant vaccines. BCG offers unique advantages as a vaccine: (1) it is unaffected by maternal antibodies and therefore it can be given at any time after birth; (2) it is usually given as a single dose eliciting a long-lasting immunity; (3) it is stable and safe; (4) it can be administrated orally; and (5) it is inexpensive to produce when compared to other live vaccines. The extraordinary adjuvant properties of mycobacteria make them an attractive vector for the development of recombinant vaccines [14]. Antigens of bacteria, parasites, and viruses have already been expressed in this attenuated mycobacterium [1, 3, 5, 11].

It has been shown that recombinant BCG (rBCG) elicits both cellular and humoral immune response against the heterologous antigens [7]. Despite of successful heterologous antigen expression and in some cases protection afforded by rBCG, instability of the recombinant vaccine during *in vitro* and *in vivo* growth has been reported [9]. This instability has been reported mainly when replicative vectors are used, and it has been implicated in failure of protection in a phase I vaccine trial in humans [8]. Integrative vectors are more stable [6], however, the disadvantage is

the lower expression level of heterologous genes compared to that of multicopy plasmids. Thus, optimization of BCG as a vehicle for live recombinant vaccines requires improved strategies for stable and high level of antigen expression. We have recently reported the use of auxotrophic complementation as a selectable marker that would be suitable for use in a recombinant vaccine [4]. A BCG auxotrophic for the amino acid leucine was constructed by knocking out the *leuD* gene by unmarked homologous recombination. Expression of *leuD* on a plasmid not only allowed complementation, but also acted as a selectable marker. Preliminary results indicate that this new system is stable maintained during *in vivo* growth, as the selective pressure is maintained.

In this report, we used the new vector system to clone and express two *Leptospira interrogans* antigens in BCG Δ*leuD.* Stability of the vector was evaluated upon inoculation of the rBCG in Golden Sirian hamsters, the standard animal model for leptospirosis. Comparison with a conventional BCG expression system was also carried out.

## MATERIAL AND METHODS

A BCG auxotrophic for the amino acid leucine, previously constructed by knocking out the *leuD* gene by unmarked homologous recombination [4] and *M. bovis* BCG Pasteur were used. Competent mycobacteria cells were prepared as follow: *M. bovis* BCG Δ*leu*D and *M. bovis* BCG Pasteur were grown in 5 ml of Middlebrook 7H9 broth (Difco) supplemented with 10% of oleic acid, albumin, dextrose complex (OADC – Difco), 0.2% glycerol and 0.05% Tween 80 (Sigma). The auxotrophic strain was grown in media supplemented with 100 μg/ml L-leucine (Sigma). When the optical density at 600 nm ($OD_{600}$) was between 0.8 and 1.0, the cultures were diluted 100-fold in the same growth medium and incubated at 37°C for 7 days in a shaker. The cultures were then incubated on ice for 1 h, harvested by centrifugation at 4,000 x *g* for 10 min at 4°C, washed three times with 10% glycerol (4°C) and resuspended in 1 ml of 10% glycerol.

The coding sequences for the *L. interrogans* antigens LipL32 and the non identical fraction of the LigA antigen (LigAni), together with the 18 kDa gene promoter from *M. Leprae*, were amplified by PCR as expression cassette from pUS2000/*lipL32*

and pUS2000/*ligAni* [13], and cloned into pUP410, an *E. coli*-mycobacteria shuttle vector that carries a kanamycin resistance gene and the *leuD* gene that allows auxotrophic complementation of the BCG Δ*leu*D strain. The fragments were ligated into the *Kpn*I site of the pUP410 vector. The resulting vectors were named pUP410+*lipL32* and pUP410+*ligAni*.

Electroporation of *M. bovis* BCG Pasteur and BCG Δ*leu*D competent cells was performed following standard methods [12]. Briefly, 100 μl electroccompetent cells were mixed with 0.5 -1 μg plasmid DNA and electroporated in 0.2 cm gap electroporation cuvettes at 2.5 kV, 25 μF and 800 Ω using a Gene Pulser II (Bio-Rad). Immediately after electroporation, the cells were diluted in 1 ml of 7H9 broth and incubated at 37°C for 24 h. *M. bovis* BCG was plated on 7H10 agar containing the appropriate antibiotic and BCG Δ*leuD* was plated on 7H10 agar without leucine. Transformants were selected after 21 days. The expression of LipL32 and LigAni in BCG was demonstrated using Western blot as previously described [13].

Four to six week-old hamsters were used to evaluate the *in vivo* stability of rBCG. A total of eighty- four animals were randomly allocated into six groups, fourteen animals per group, and inoculated with approximately $10^6$ CFU of rBCG in 100 μl of sterile PBS-T. Group one was inoculated with rBCG Δ*leuD* (pUP410+*lipL32*), group two with rBCG Δ*leuD* (pUP410), group three with rBCG Pasteur (pUP410+*lipL32*), group four with rBCG Pasteur (pUP410), group five with rBCG Δ*leuD* (pUP410+*ligAni*), and group six with rBCG Pasteur (pUP410+*ligAni*) . At week 2, 5, 8, 12, 16 and 18 after inoculation the spleen from two hamsters per group was removed, homogenized, serially diluted in 7H9, and plated onto 7H10 with or without selection (L-leucine for rBCG Δ*leu*D, and kanamycin for rBCG Pasteur). The number of resultant colonies was compared.

## RESULTS AND DISCUSSION

Auxotrophic BCG strains, when inside macrophages, are unable to access intracellular amino acids, thus they fail to grow [2]. This important observation opened the possibility of using auxotrophic complementation as a selectable marker, an approach that has two main advantages: it provides active selection *in vivo*, unlike antibiotic resistance markers, and it abolishes the need for using an antibiotic

resistance gene as a vector component. Improved stability of a plasmid vector that uses auxotrophic complementation as a selectable marker in recombinant BCG has already been reported in mice [4]. This system is based on the use of a strain of *M. bovis* BCG auxotrophic for the leucine amino acid as a result of an unmarked mutation in the leuD gene. Complementation is achieved with the presence of the gene *leuD* inserted into the plasmid vector, which acts as a selectable marker.

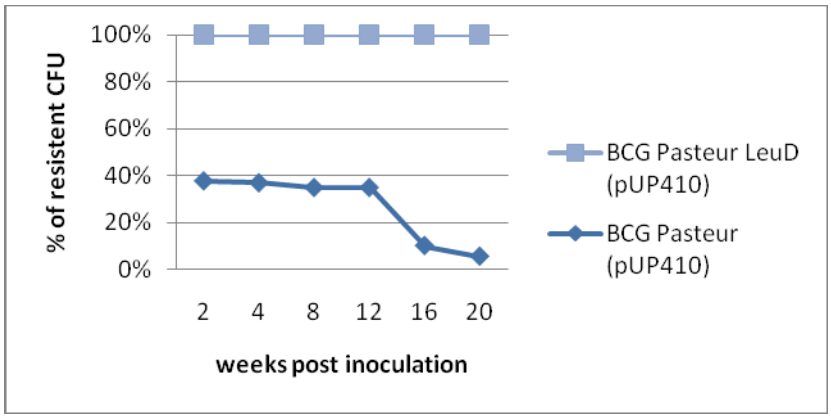
Cloning of the coding sequence of *lipL32* and *ligAni* fused to the *in vivo* induced *M. leprae* 18kDa gene promoter [6] in the auxotrophic complementation vector pUP410 resulted in the vectors named pUP410+*lipL32* and pUP410+*lig*Ani, respectively. These were used to transform the strain of *M. bovis* BCG leuD and *M. bovis* BCG Pasteur. Expression of LipL32 or LigAni in BCG was demonstrated by WB using MAbs against LipL32 or LigAni. These MAbs recognized a protein of approximately 30 kDa in rBCG cell lysates expressing LipL32 and approximately 60 kDa in rBCG cell lysates expressing LigAni. In contrast, no band was detected in wild type BCG extract, demonstrating the specificity of these antibodies. Based on band intensities, the level of expression of rLipL32 or LigAni in recombinant BCG grown *in vitro* was considered to be similar (data not show). This information is relevant for subsequent studies of immune protection that will be carried out.

Stability of *M. bovis* BCG Δ*leu*D and *M. bovis* BCG Pasteur transformed with plasmids containing the *leuD* gene as selectable marker was evaluated in hamsters. Initial selection was carried out on medium without leucine for BCG Δ*leu*D or containing kanamycin for BCG Pasteur. Approximately $10^6$ CFU of each strain were inoculated into hamsters. Bacteria were recovered from spleen of two animals killed at 2, 5, 8, 12, 16 and 18 weeks post inoculation and plated on selective and non-selective medium. Ratios of resistant (rBCG) versus total BCG colonies were calculated for each strain. Average of the BCG Δ*leuD* and BCG Pasteur strains is shown in Figure 1. Auxotrophic complementation vectors used to transform BCG Δ*leu*D showed 100% of stability *in vivo* during the 18 week-experiment (Figure 1). Conversely, the same vectors transformed in BCG Pasteur were lost during *in vivo* growth. Two weeks post inoculation less than 50% of the bacterial cells still had the plasmid. This number fell to 7% at 18 weeks post inoculation (Figure 1). Interestingly, it was noted that there was no significant reduction in the number of CFU recovered from hamsters inoculated with BCG Δ*leuD* complemented strains in

comparison to rBCG Pasteur, showing that auxotroph complementation does not come at the cost of decreased rBCG survival. These results are similar to those obtained when the same plasmid vector expressing the *lacZ* gene was evaluated in mice [4], suggesting that the stability is not dependent on the foreign gene or the animal species used.

The use of auxotrophic complementation as selectable marker for expression of foreign genes in BCG described in this report provides the high levels of stability previously described only for integrative vectors [10], with the advantages of a multicopy vector, namely higher levels of foreign antigen expression. Although the kanamycin resistance gene was still present in the vectors used in this study, it can be removed by digestion with *Hind*III, followed by religation using conditions that favour intramolecular ligation. The functionality of this approache has already been demonstrated [4]. Evaluation of the immune response obtained with the use of this new selection system is currently being performed. It is expected that it will be able to elicit a strong and long-lasting immune response against these antigens, contributing to the development of BCG into a vaccine able to protect also against leptospirosis.

A

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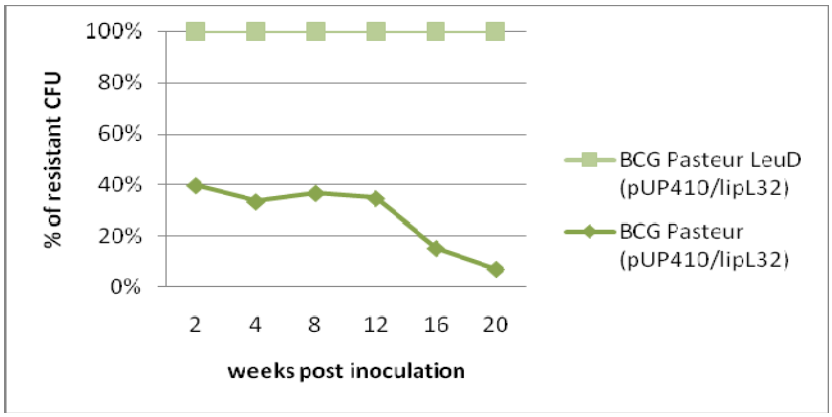


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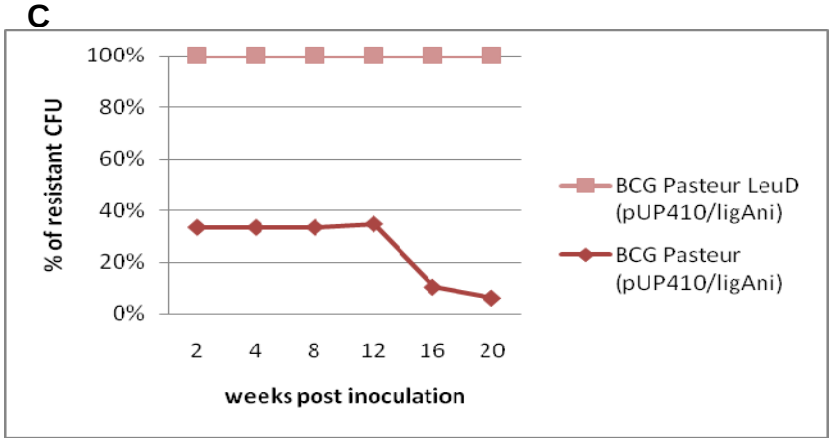


**FIGURE 1.** *In vivo* stability of rBCG transformed with auxotrophic complementation vectors. Hamsters were inoculated with BCG Δ*leu*D transformed with pUP410, pUP410+*lipL32* and pUP410+*ligAni* and BCG Pasteur transformed with the same vectors. Bacteria were recovered from spleen of two animals from each group killed at 2, 4, 8, 12, 16 and 20 weeks post inoculation and plated on selective and non-selective medium. Ratios of resistant (rBCG) versus total BCG colonies were calculated for each strain and construction (A, B and C).

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# 6 CONCLUSÕES

- O antígeno LipL32, nas formas de vacina de DNA, de subunidade recombinante e BCG recombinante, é capaz de induzir resposta imune específica em camundongos;

- LipL32, presente na superfície de Leptospira, é reconhecida por anticorpos presentes no soro de camundongos imunizados com as diferentes construções ;

- BCG expressando LipL32 protege hamsters frente ao desafio letal com uma cepa virulenta de *L. interrogans*;

- O sistema de clonagem em *M. bovis* BCG que utiliza complementação auxotrófica como marcador de seleção permite a manutenção estável do vetor plasmidial após a inoculação em hamster, enquanto o sistema convencional é instável sem pressão de seleção.

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