

# Recombinant esterase from *Corynebacterium pseudotuberculosis* in DNA and subunit recombinant vaccines partially protects mice against challenge

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## Abstract

**Purpose.** We tested the efficacy of the esterase encoded by *cp1002\_RS09720* from *Corynebacterium pseudotuberculosis* in recombinant subunit and DNA caseous lymphadenitis (CLA) vaccines. This target was predicted as one of the best CLA vaccine candidates by mature epitope density analysis.

**Methodology.** Gene *cp1002\_RS09720* was cloned into two different vectors (pAE for subunit vaccine and pTARGET for DNA vaccine). Four groups of 15 mice each were immunized with the recombinant esterase rCP09720 associated with aluminium hydroxide adjuvant (G1), pTARGET/*cp09720* DNA vaccine (G2), a naked pTARGET (G3) or PBS as a negative control (G4). Immunization occurred in two doses intercalated by a 21 day interval. Twenty-one days after the last dose administration, animals were challenged with a virulent *C. pseudotuberculosis* MIC-6 strain.

**Results.** G1 showed high levels of IgG1 and IgG2a on days 21 and 42 post-immunization and a significant level of IFN- $\gamma$  ( $P < 0.05$ ), suggesting a Th1 response. The protection levels obtained were 58.3 and 16.6 % for G1 and G2, respectively.

**Conclusion.** The subunit vaccine composed of the recombinant esterase rCP09720 and Al(OH)<sub>3</sub> is a promising antigenic formulation for use against CLA.

## INTRODUCTION

*Corynebacterium pseudotuberculosis*, the aetiological agent of caseous lymphadenitis (CLA) in sheep and goats, is a Gram-positive, aerobic and facultative intracellular bacteria that preferably infects macrophages [1]. This bacterium affects a wide variety of hosts, including small ruminants, cows, horses and wild animals [2]. It can even infect humans through the contamination of meat and milk, and the infection of veterinarians and farm practitioners [3]. It is a globally distributed chronic and subclinical disease, with high prevalence in developing countries [4], and it has been pointed out as one of the main causes of economic losses in Africa and South America [5]. CLA treatment is not effective

because the bacterium is located inside encapsulated pyogranulomas and cannot be reached by antibiotics [6].

Vaccination is the most important approach for CLA control since animals are refractory to therapeutics, due to antibiotics' inability to reach the bacteria inside abscesses [7]. Several vaccination strategies have been proposed, including inactivated or attenuated *C. pseudotuberculosis* strains and native antigens from culture supernatants [5, 8–10]. One of the most studied vaccinal targets is phospholipase D (PLD), a major *C. pseudotuberculosis* virulence factor responsible for its dissemination in the host and demonstrated to provide partial protection when used as a vaccine in some animal models [11, 12]. Commercial CLA vaccines are based on attenuated strains of *C. pseudotuberculosis* and are not

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**Keywords:** caseous lymphadenitis; *Corynebacterium pseudotuberculosis*; DNA vaccine; recombinant subunit vaccine.

**Abbreviations:** CHO, Chinese hamster ovary; CLA, caseous lymphadenitis; IFA, immunofluorescence assay; i.m., intramuscularly; MED, mature epitope density; PLD, phospholipase D; s.c., subcutaneously.

able to fully protect susceptible animals, cannot be used in all host species, present low efficacy in goats, and are not licensed for use in many countries [7]. Additionally, side effects are associated with commercially available vaccines such as fever, malaise, reductions in milk production and distribution of lesions, being more intense in goats [7, 9].

*C. pseudotuberculosis* genome sequence analysis provided critical data necessary to elucidate the molecular and genetic basis of this bacterium's virulence, and it is also useful for predicting new vaccine targets [13–16]. This approach allowed the development of new DNA and recombinant subunit vaccines based on recently discovered antigenic targets [17, 18]. A few recombinant antigens have been tested as immunogens against *C. pseudotuberculosis* infection, with limited success [18–20].

Esterase, encoded by the *cp1002\_RS09720* gene, recently reannotated (old tag: *cp1002\_1957*), was first predicted as a potential vaccine target in a pan-secretome study of *C. pseudotuberculosis* 1002 and C231 strains [21]. Through mature epitope density (MED) analysis, a computational strategy based on measuring epitope concentration in a mature protein, this protein was ranked as one of the best targets for use in CLA vaccines or diagnostic trials [21]. In fact, the recombinant esterase rCP09720 was used in an ELISA-based system with the objective of detecting the infection by *C. pseudotuberculosis* in sheep, presenting satisfactory levels of specificity and sensitivity [22]. Despite its importance, this target has never been used in vaccine formulations before. Here, we assessed the potential of the recombinant esterase as a DNA and subunit vaccine against *C. pseudotuberculosis* infection in a murine model. In addition, humoral and cellular immune responses induced by recombinant vaccines were characterized.

## METHODS

### Strains and culture conditions

The sequenced strains of *C. pseudotuberculosis* 1002 [23] and MIC-6 strains (already sequenced, currently at the annotation and assembly phases; personal communication by Vasco Azevedo), *Escherichia coli* TOP10 (Invitrogen) and *E. coli* BL21 Star (Invitrogen) cells were used in this study. *C. pseudotuberculosis* strains were cultivated in brain heart infusion (BHI) broth (Acumedia), supplemented with 0.5% Tween 80 at 37 °C for 72 h in a shaker at 200 r.p.m., or in BHI agar 1.5% [24]. *E. coli* strains were grown in Luria–Bertani (LB) medium or LB agar 1.5% for 16 h at 37 °C. When necessary, LB medium was supplemented with ampicillin at 100 µg ml<sup>-1</sup>.

### Cloning of the *cp1002\_RS09720* gene from *C. pseudotuberculosis*

Amplification of the *cp1002\_RS09720* gene was performed as described previously [25] using primers F 5' ACC ATG GGG CCT CGC GAC TGG CTG CGC 3' and R 5' CCG GAA TTC TTA CCA GGC GTT CAT AAC GT 3', designed based on the *C. pseudotuberculosis* 1002 sequence (GenBank accession no. WP\_013242814.1) using Vector NTI 10 software

(Invitrogen). For PCR, 50 ng *C. pseudotuberculosis* strain 1002 genomic DNA was added to 10 µM of each primer and PCR Mastermix (Promega) in a final volume of 50 µl. PCR product was visualized in 1% agarose gel stained with Blue Green (LGC Biotechnology). Gene *cp1002\_RS09720* was cloned into pAE plasmid [26]. Thus, pAE vector and the *cp1002\_RS09720* gene were digested with the restriction enzymes *Bam*HI and *Eco*RI (Invitrogen), and ligation was performed using T4 DNA ligase (Invitrogen). The pAE/cp09720 recombinant clone was characterized by restriction enzyme digestion and DNA sequencing.

For DNA vaccine construction, the *cp1002\_RS09720* gene was amplified using the primers F 5' ACC ATG GGG CCTC GCG ACT GGC TGC GC and R 5' CCG GAA TTC TTA CCA GGC GTT CAT AAC GT 3'. Primers were designed based on the *C. pseudotuberculosis* 1002 sequence (GenBank accession no. NC\_017300.1) using Vector NTI 10 software (Invitrogen). The amplicon was inserted into the pTARGET vector (Promega) following the manufacturer's instructions. Recombinant vectors were selected with 100 µg ml<sup>-1</sup> ampicillin and 20 mg ml<sup>-1</sup> X-Gal (Invitrogen). The recombinant vector was denominated pTARGET/cp09720.

### Expression and purification of recombinant protein rCP09720

The expression and purification of rCP09720 were performed as described previously [25]. Briefly, plasmid pAE/cp09720 was inserted in *E. coli* BL21 (DE3) Star. IPTG (1 mM) was added to induce protein expression in an orbital shaker at 37 °C for 3 h. Expression of rCP09720 was confirmed by Western blotting using a peroxidase conjugated monoclonal antibody against 6×His tag (Sigma). Nickel-affinity chromatography purification was performed on a sepharose column (HisTrap; GE Healthcare). The purity level was determined by 12% SDS-PAGE, and protein concentration using a commercial kit based on bicinchoninic acid methodology (Pierce).

### *In vitro* transfection of Chinese hamster ovary cells with DNA vaccine

Chinese hamster ovary (CHO) cells were transfected with DNA from pTARGET/cp09720 or the naked pTARGET to confirm *in vitro* protein expression. CHO cells were cultivated on polystyrene 96-well plates (Nunc) using Dulbecco's modified Eagle's medium (Invitrogen) medium as described previously [27]. After an 80% confluence, cells were transfected using 1 µg pTARGET/cp09720 or pTARGET and 1 µl Lipofectamine 2000 (Invitrogen). After 48 h of incubation, protein expression was evaluated using immunofluorescence assay (IFA). Polyclonal antibodies directed against the rCP09720 protein (1:100) and rabbit anti-mouse antibody conjugated with fluorescein isothiocyanate (FITC; Sigma-Aldrich) diluted at 1:80 were used for IFA.

### Experimental animals and ethical aspects

BALB/c mice (6–8 weeks old) were obtained from the Central Animal Facility of the Federal University of Pelotas, where the immunization assay was also conducted. All mice were kept in

cages containing wood shavings, bedding, free access to water and a maintenance diet *ad libitum* in a 12 h light/dark cycle, at room temperature of  $21 \pm 2^\circ\text{C}$ . All experiments were performed in compliance with the procedures of the Brazilian College of Animal Experimentation (COBEA). The Ethics Committee on Animal Experimentation (CEEA) of Pelotas Federal University (UFPEL) approved the project (approval number 2442). All efforts were made to maximize animals' welfare.

### Vaccination and challenge

BALB/c mice (6–8 weeks old) were divided into four groups of 15 mice each. Animals were immunized with two doses intercalated by 21 day intervals. Groups were distributed as follows: G1, subcutaneously (s.c.) inoculated with 50  $\mu\text{g}$  of rCP09720 associated with  $\text{Al}(\text{OH})_3$  at 15%; G2, intramuscularly (i.m.) inoculated with 50  $\mu\text{g}$  of DNA vaccine pTARGET/cp09720; G3, i.m. inoculated with naked pTARGET; and G4, control, s.c. inoculated with PBS. Blood samples were collected by retro-orbital plexus puncture on days 0, 21 and 42 after the first immunization. Twelve mice per group were challenged intraperitoneally (i.p.) 21 days after the last immunization with  $10^4$  c.f.u. of *C. pseudotuberculosis* MIC-6 virulent strain [20]. Then, mice were monitored daily for 30 days and deaths recorded. The protocol of DNA vaccination was performed according to [18] and subunit vaccination according to [28].

### Assessment of humoral immune response

IgG1 (related to a Th2 cellular response) and IgG2a (related to a Th1 cellular response) levels in individual mice serum samples were determined by indirect ELISA, as described previously [29] with minor modifications. High binding polystyrene 96-well plates (Maxisorp; Nunc) were coated with 100  $\text{ng well}^{-1}$  of the rCP09720 recombinant protein (1  $\mu\text{g ml}^{-1}$ ) diluted in bicarbonate-carbonate buffer (pH 9.6) for 16 h at  $4^\circ\text{C}$ . After that, plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T). Noncoated binding sites were blocked with 100  $\mu\text{l well}^{-1}$  of 5% skimmed milk diluted in PBS for 1 h at  $37^\circ\text{C}$ . Serum samples were diluted 1:100 in PBS-T and incubated for 1 h at  $37^\circ\text{C}$ , and washed three times with PBS-T. Then, 100  $\mu\text{l well}^{-1}$  of goat anti-mouse IgG1 or IgG2a (Sigma-Aldrich) was added (1:4000) for 1 h at  $37^\circ\text{C}$  and washed five times with PBS-T. Then, rabbit anti-goat IgG conjugated with horseradish peroxidase (Sigma-Aldrich) diluted in PBS-T (1:6000) was added. After incubation for 1 h at  $37^\circ\text{C}$ , plates were then washed five times with PBS-T. A colorimetric reaction was obtained using substrate-chromogen solution (*o*-phenylenediamine dihydrochloride, OPD; Sigma-Aldrich) in 0.4  $\text{mg ml}^{-1}$  phosphate-citrate buffer containing 0.04% 30% hydrogen peroxide (pH 5.0). Plates were incubated at room temperature in the dark for 15 min. The reaction was stopped by adding 25  $\mu\text{l}$  4 N  $\text{H}_2\text{SO}_4$ . Absorbance values were obtained on an ELISA plate reader (Mindray) set at 450 nm. Each sample was tested in duplicate. The best dilutions for sera and conjugate and antigen concentration were achieved by a previous checkerboard titration.

### Assessment of cellular immune response

Cellular immune response evaluation was performed as previously described by *C. pseudotuberculosis* research protocols in mice models [30], with some modifications. Briefly, at 21 days from the last immunization, three mice from groups G1, G2 and G4 were sacrificed, and the spleen removed for splenocyte isolation. Spleen cell suspensions obtained from each animal were placed in complete RPMI medium [RPMI 1640 medium (Gibco BRL) supplemented with 10% foetal bovine serum (Sigma-Aldrich), 100 IU  $\text{ml}^{-1}$  penicillin and 100  $\mu\text{g ml}^{-1}$  streptomycin (LGC Bio)], counted in a Neubauer chamber and cell concentration was adjusted to  $2 \times 10^6$  cells  $\text{ml}^{-1}$ . Viability was assured by exclusion with Trypan Blue staining (Vetec). Splenocytes were cultivated in triplicate into 24-well plates (Techno Plastic Products) containing  $2 \times 10^5$  cells (100  $\mu\text{l}$ ) per well. Cells were stimulated with sterile PBS (negative control), 15  $\mu\text{g ml}^{-1}$  recombinant protein rCP09720 or 8  $\mu\text{g ml}^{-1}$  concanavalin A (ConA) mitogen (positive control). Plates were incubated with 5%  $\text{CO}_2$  at  $37^\circ\text{C}$  for 72 h (maximum). Splenocytes were then collected and submitted to total RNA extraction with Trizol (Invitrogen), following the manufacturer's recommendations. Synthesis of cDNA was performed with 1  $\mu\text{g}$  total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's recommended protocol. The cDNA was used for quantification of the expression profile of the cytokines IL-4, IL-10, IL-12 and IFN- $\gamma$  by real-time PCR using the Stratagene Mx3005P Real-Time PCR System (Agilent Technologies), and Sybr Green reagent (Applied Biosystems). Primers sequences used were F 5' CCAAGGTGCTTCGCATA TTT 3' and R 5' ATCGAAAAGCCCCGAAAGAGT 3' for IL-4 [31] F 5' TTTGAATTCCTGGGTGAGAA 3' and R 5' ACAGGGGAGAAATCGATGACA 3' for IL-10 [32], F 5' AGCACCAGCTTCTTCATCAGG 3' and R 5' CCTTTC TGGTTACACCCCTCC 3' for IL-12 [32], F 5' GCGTCA TTGAATCACACCTG 3' and R 5' TGAGCTCATTGAA TGCTTGG 3' for IFN- $\gamma$  [31]. The results were normalized using the GPDH gene using the primers F 5' AACGACCCC TTCATTGAC 3' and R 5' TCCACGACATACTCAGCAC 3', as described previously [33].

### Statistical analysis

Data were expressed as the mean  $\pm$  SD and analysed using GraphPad Prism, version 5, for Windows (GraphPad Software). Statistical differences among groups for IgG1 and IgG2a levels and cytokine expression were determined using one-way ANOVA followed by Tukey's post-test. Fisher's test and a log-rank test were used to determine significant differences in mortality and survival rates, respectively. Data were considered statistically different when the *P*-value was 0.05 or less.

## RESULTS

### Recombinant vaccines

Recombinant esterase rCP09720 from *C. pseudotuberculosis* was successfully expressed in *E. coli* BL21 (DE3) Star strain. The resultant yield after purification and refolding was

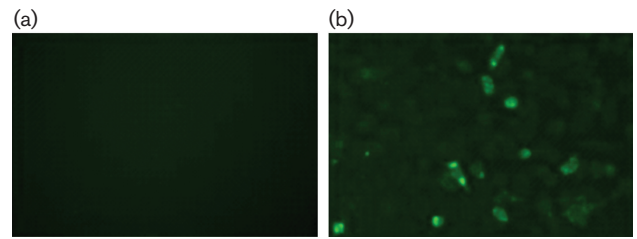
6 mg l<sup>-1</sup> recombinant protein. Expression was confirmed by Western blot analysis using a monoclonal anti-6×His antibody (Fig. 1). The DNA vaccine and a control plasmid (pTARGET/cp09720 and pTARGET) were used to transfect CHO cells to assess *in vitro* CP1957 expression (Fig. 2). *In vitro* expression of the CP1957 protein was confirmed by IFA and the clone with more intense fluorescence was selected as the DNA vaccine.

**Humoral immune response induced in immunized mice**

Recombinant protein-based vaccine rCP09720/Al(OH)<sub>3</sub> (G1) induced the highest levels of IgG1 and IgG2a, as shown in Fig. 3. G1 was capable of inducing a significantly higher (*P*<0.05) production of IgG1 (Fig. 3a) and IgG2a (Fig. 3b) when compared to all other groups (G2, G3 and G4) on days 21 and 42 after immunization.

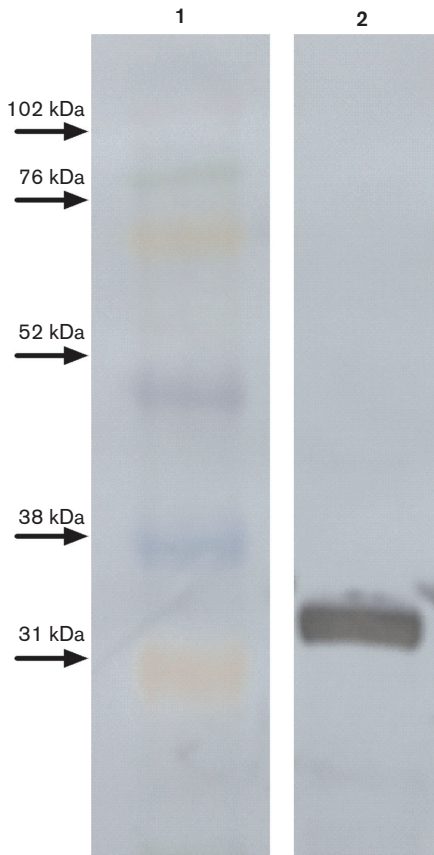
**Cellular immune response evaluation**

Cellular immune response was assessed by the transcription levels of genes that encode cytokines IL-4, IL-10, IL-12 and IFN-γ (Fig. 4). The results showed that G1 and

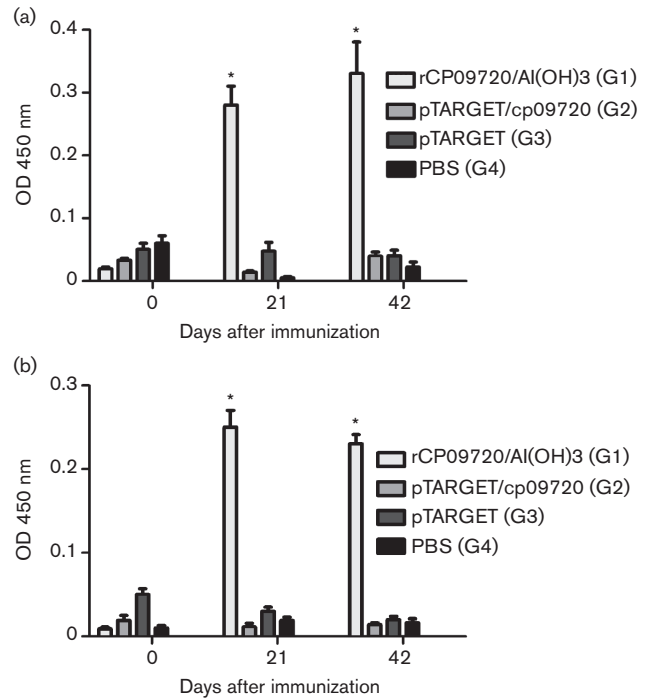


**Fig. 2.** Indirect immunofluorescence of CHO cells transfected with (a) pTARGET control and (b) pTARGET/cp1957, using a polyclonal anti-rCP09720, revealed the *in vitro* expression of the CP09720 protein.

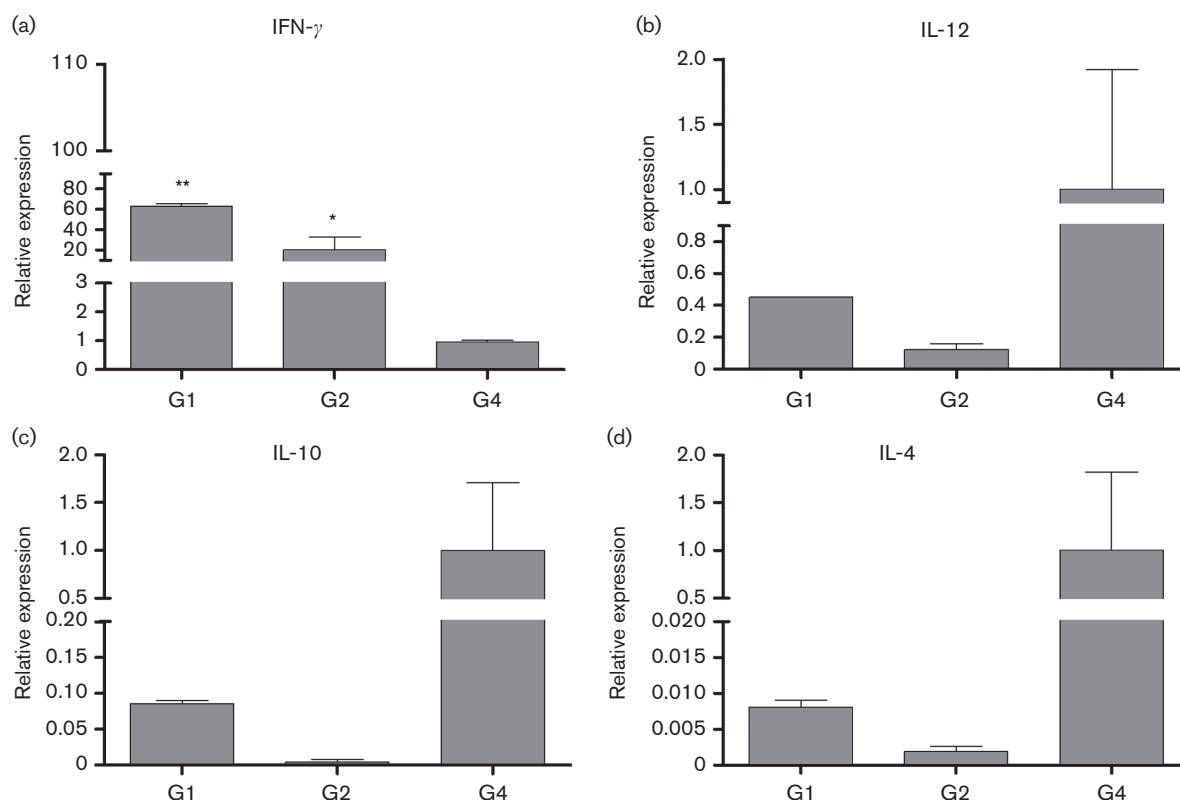
G2 were able to induce significantly increased levels of IFN-γ expression when compared to the G3 and G4 control groups (*P*<0.05). However, G1 presented the highest levels of IFN-γ mRNA (Fig. 4a). No significant levels of IL-12, IL-10 and IL-4 were observed in the G1 and G2 groups when compared to the G4 control group, and no significant statistical differences were observed when G1 and G2 were compared (Figs 4b–d).



**Fig. 1.** Western blot analysis using monoclonal anti-6×His antibody (1 : 4000) showing the purified rCP09720 of approximately 34 kDa used for vaccine preparation. 1, rCP09720 protein. 2, Full-Range Rainbow Molecular Weight Marker (GE Healthcare).



**Fig. 3.** IgG1 (a) and IgG2a (b) levels induced by recombinant subunit and DNA vaccines developed with the target CP09720 of *C. pseudotuberculosis*. The measurement was performed by indirect ELISA at 0, 21 and 42 days post-immunization, and results were obtained in an ELISA reader set at 450 nm and expressed as optical density values. Results are presented as means and standard deviation (bars) for each experiment with 12 animals per group. Significant differences between IgG isotype production in different groups was calculated by employing one-way ANOVA followed by the Tukey post-test. \*Result statistically different (*P*<0.05) in relation to negative control (G4).



**Fig. 4.** Relative expression of IFN- $\gamma$ , IL-12, IL-10 and IL-4, induced in splenocyte cells taken from mice immunized with the vaccine formulations and their controls (at 21 days from the last immunization) quantified by real-time PCR. Results presented as mean and standard error. (G1) rCP09720/Al(OH)<sub>3</sub>, (G2) pTARGET/cp09720 and (G4) PBS vaccine groups. \*Significantly higher results ( $P < 0.05$ ) compared to the other experimental groups from the one-way ANOVA and Tukey post-test. \*\*Significantly higher results ( $P < 0.05$ ) compared to controls and the other experimental groups from one-way ANOVA and Tukey post-test.

### Protective potential of recombinant esterase

Immunization with rCP09720/Al(OH)<sub>3</sub> (G1) partially protected animals against infection with *C. pseudotuberculosis* MIC-6 strain ( $P < 0.05$ ), resulting in a protection rate of 58.3 % (Fig. 5). As regards the DNA vaccine, G2 immunized with pTARGET/cp09720 showed a lower protection rate (16.6 %,  $P > 0.05$ ), despite the increased survival rate for G3 or G4. All the animals in the negative control groups (G3 and G4) died. No side effects, tissue damage or any modification of animals' health conditions due to the vaccination protocol or even due to the vector herein used was observed.

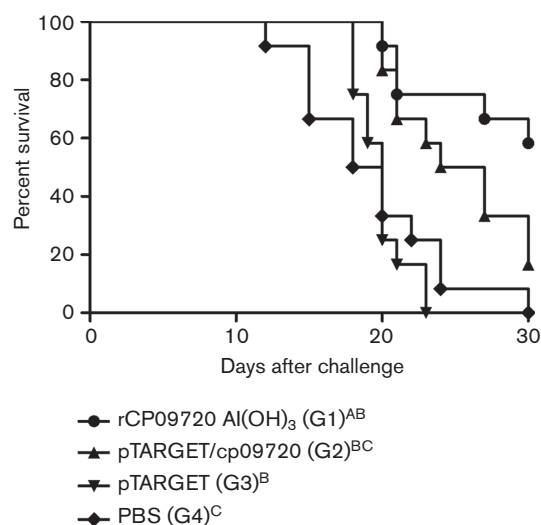
### DISCUSSION

To our knowledge, this is the first work describing the use of the *cp1002\_RS09720* gene from *C. pseudotuberculosis* in recombinant subunit and DNA vaccines. The gene was recently described as an immunogenic target, with a high number of epitopes in the mature protein, by using the MED approach [22]. It is important to highlight that *cp1002\_RS09720* is a highly conserved gene, presenting 100 % identity among 31 sequenced strains of *C. pseudotuberculosis*

(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), which makes it an excellent target for vaccines in field conditions.

Here, the formulation based on rCP09720 associated with Al(OH)<sub>3</sub> was able to confer a significant protection level ( $P < 0.05$ ) against challenge with the *C. pseudotuberculosis* MIC-6 virulent strain. The protective potential in G1 was associated with the highest levels of IgG1 and IgG2a, significant production of IFN- $\gamma$ , and low biological levels of IL-4 and IL-10, a situation that points to an activation directed to the Th1 cellular immune profile. Aluminium hydroxide is the most used adjuvant for vaccine formulations. It is well known for its significant Th2 activation and induction of high levels of antibody production [34]. However, in some cases, aluminium hydroxide can drive a Th1 response in mice models, depending on the antigen employed in the vaccine's design [35]. In this study, we could observe the last situation, since a significant increase in specific IgG1 production (associated with a Th2 response in mice) was observed [36]. Other adjuvants have been used in CLA vaccine formulation [3]. For example, levamisole, which is known for inducing a T helper type 1 response, was used in combination with a toxoid vaccine [37]. A study was carried





**Fig. 5.** Survival curve of vaccinated animals using recombinant subunit and DNA vaccines after challenge with  $10^4$  c.f.u. of an MIC-6 virulent strain of *C. pseudotuberculosis*. Animals were observed for 30 days. Survival curves with different letters are statistically significant in Fisher's exact test ( $P < 0.05$ ).

out to examine the combined effect of vaccination with a commercial vaccine, and the results showed that the zinc-combined vaccine, as well as a washed antiseptic, were safe for ewes [38]. The muramyl dipeptide (MDP), an immune stimulant of the reticuloendothelial system, was used in association with low and high doses of crude bacterial antigens, cell wall and toxin. The authors concluded that vaccines composed of high doses of *C. pseudotuberculosis* toxin associated with MDP showed induction of protective immunity in alpacas challenged with a virulent *C. pseudotuberculosis* strain [39].

Despite the partial protection (58.3%) offered by the rCP09720/Al(OH)<sub>3</sub> vaccine formulation, it should be considered that a few studies have been conducted to develop a recombinant subunit vaccine for CLA and most showed lower and intermediate rates. The use of rHSP60 did not protect mice against a virulent *C. pseudotuberculosis* challenge [18, 40]. Only 44% of sheep immunized with genetically inactivated PLD were protected against CLA [11]. A recombinant serine protease of 40 kDa (rCP40) associated with saponin was able to protect 90% of challenged mice [20]. When rCP40 was associated with Freund's adjuvant, 100% of animals were protected after challenge. However 60% of animals immunized with the control Freund's adjuvant survived [41].

The use of pTARGET/cp09720 as a DNA vaccine was not able to confer significant protection (16.6% protection rate,  $P > 0.05$ ) and to induce IgG1 or IgG2a production. However, immunization with pTARGET/cp09720 induced significant IFN- $\gamma$  production ( $P < 0.05$ ), which can be associated with the rate of protection. DNA vaccine is known to induce

a Th1 cellular response mainly by the induction of IFN- $\gamma$  production by CD4+ Th1 lymphocytes and CD8+ cytotoxic T cells [36, 42].

Previous studies described DNA vaccines as less effective than conventional or protein subunit vaccines in protecting against infection when considering some specific pathogen models, probably due to the weak and short-term immunity elicited [1, 43]. So far, just two targets have been assessed in DNA vaccines against *C. pseudotuberculosis* [44]. *hsp60* DNA vaccination in mice was not able to induce protection [18], but when a genetically detoxified phospholipase D ( $\Delta$ PLD) was targeted as a CTLA-4 fusion protein, vaccination provided a 70% protection level [44]. In our study, pTARGET/cp09720 was not able to induce a specific immune response nor a significant protection against CLA in mice, and maybe a technique aiming to target proteins to antigen-presenting cells could be capable of increasing the immune potential of DNA vaccines with rCP09720.

Subunit and DNA recombinant vaccines have been developed in an attempt to improve the efficacy of CLA vaccines. The route of administration is important when these kinds of vaccines are used. For example, de Rose *et al.* [45] tested and proved the superiority of i.m. compared to s.c. in inducing a strong memory response and sterile immunity following challenge with *C. pseudotuberculosis* through the use of a plasmid encoding a PLD antigen linked to CTLA4-Ig. However, the s.c. route is the more utilized in all kinds of CLA vaccine (toxoid, bacterin, subunit recombinant and DNA vaccines) [3].

Several models of CLA vaccines have already been evaluated [3, 30]. The first kind of vaccine evaluated for CLA prevention was killed whole bacteria (bacterin). However, the protection level was only partial [46, 47]. The secreted toxoid of *C. pseudotuberculosis* (PLD) is another approach for CLA vaccines [48, 49]. Some studies have used a bacterin and toxoid combination [37, 50, 51]. A study of natural infection in goats using this kind of vaccine was not completely efficient and it was recommended only as a protective measure against CLA [50]. The strategy used in our study was also evaluated in other studies, but few antigens were assessed using this strategy and they resulted in variable protection levels [20, 40, 52].

The Th1 response is considered to be the protective immunity against *C. pseudotuberculosis* infection [44, 53]. Moreover, the major cytokine involved in the effector mechanisms of this immune response is IFN- $\gamma$ . This cytokine enhances phagocytosis activity in macrophages and activates CD8+ cytotoxic cells to destroy infected cells [52, 54]. IL-12 also has an important role in this process because it can be produced by antigen-presenting cells and drive Th0 cells to differentiate into Th1 effector cells. In our study, recombinant esterase rCP09720 was clearly able to induce a Th1 response due to the significant IFN- $\gamma$  production, high IgG2a levels and low biological levels of IL-10 and IL-4. However, this immune response was not able to confer

full protection against the virulent bacteria. This points towards the following considerations. First, although the antigen activates an adequate cellular immune response, the response intensity was not sufficient to quell infection by an extremely virulent *C. pseudotuberculosis* strain. Second, since the antigen can activate a Th1 response, we could manipulate the immune response through different antigen doses, adjuvants, route of administration and time of vaccination to induce more intense lymphocyte activation and cytokine and immunoglobulin production. Our group is searching for new vaccinal methodologies to enhance the protection conferred by the recombinant esterase.

In conclusion, the recombinant esterase rCP09720 associated with an Al(OH)<sub>3</sub> adjuvant protected 58.3% of mice against *C. pseudotuberculosis* virulent challenge. It induced a humoral response and an adequate, but not sufficient, Th1 response required for full protection. On the other hand, DNA vaccination with pTARGET/cp09720 showed 16.6% protection in challenged mice. It induced IFN- $\gamma$  production but failed to induce anti-rCP09720-specific antibodies.

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#### Conflicts of interest

The authors declare that there are no conflict of interest.

#### References

- Baird GJ, Fontaine MC. *Corynebacterium pseudotuberculosis* and its role in ovine caseous lymphadenitis. *J Comp Pathol* 2007;137:179–210.
- Dorella FA, Fachin MS, Billault A, Dias Neto E, Soravito C et al. Construction and partial characterization of a *Corynebacterium pseudotuberculosis* bacterial artificial chromosome library through genomic survey sequencing. *Genet Mol Res* 2006;5:653–663.
- Bastos BL, Portela RWD, Dorella FA, Ribeiro D, Seyffert N et al. *Corynebacterium pseudotuberculosis*: immunological responses in animal models and zoonotic potential. *J Clin Cell Immunol* 2012;S4–S5.
- Guimarães AS, Carmo FB, Heinemann MB, Portela RW, Meyer R et al. High sero-prevalence of caseous lymphadenitis identified in slaughterhouse samples as a consequence of deficiencies in sheep farm management in the state of Minas Gerais, Brazil. *BMC Vet Res* 2011;7:68.
- Ruiz JC, D'Afonseca V, Silva A, Ali A, Pinto AC et al. Evidence for reductive genome evolution and lateral acquisition of virulence functions in two *Corynebacterium pseudotuberculosis* strains. *PLoS One*;6:e18551.
- Radusky LG, Hassan S, Lanzarotti E, Tiwari S, Jamal S et al. An integrated structural proteomics approach along the druggable genome of *Corynebacterium pseudotuberculosis* species for putative druggable targets. *BMC Genomics* 2015;16:S9.
- Dorella FA, Pacheco LG, Seyffert N, Portela RW, Meyer R et al. Antigens of *Corynebacterium pseudotuberculosis* and prospects for vaccine development. *Expert Rev Vaccines* 2009;8:205–213.
- Simmons CP, Dunstan SJ, Tachedjian M, Krywult J, Hodgson AL et al. Vaccine potential of attenuated mutants of *Corynebacterium pseudotuberculosis* in sheep. *Infect Immun* 1998;66:474–479.
- Meyer R, Carminati R, Cerqueira RB, Vale V, Viegas S et al. Evaluation of humoral immune response in goats inoculated with a live attenuated lyophilized vaccine against *Corynebacterium pseudotuberculosis*. *Rev Ciênc Méd Biol* 2002;1:42–48.
- Lima DD, Rebouças MF, Portela RW, Vale V, Pacheco A et al. Comparison of two chromogens in the development of an immunoenzymatic assay (ELISA) for detection of immunoglobulins specific against *Corynebacterium pseudotuberculosis*. *Vet Immunol Immunopathol* 2009;128:333.
- Hodgson AL, Carter K, Tachedjian M, Krywult J, Corner LA et al. Efficacy of an ovine caseous lymphadenitis vaccine formulated using a genetically inactive form of the *Corynebacterium pseudotuberculosis* phospholipase D. *Vaccine* 1999;17:802–808.
- Menziés PI, Hwang YT, Prescott JF. Comparison of an interferon- $\gamma$  to a phospholipase D enzyme-linked immunosorbent assay for diagnosis of *Corynebacterium pseudotuberculosis* infection in experimentally infected goats. *Vet Microbiol* 2004;100:129–137.
- Hassan SS, Guimarães LC, Pereira UP, Islam A, Ali A et al. Complete genome sequence of *Corynebacterium pseudotuberculosis* biovar Ovis strain P54B96 isolated from antelope in South Africa obtained by rapid next generation sequencing technology. *Stand Genomic Sci* 2012;7:189–199.
- Silva A, Schneider MP, Cerdeira L, Barbosa MS, Ramos RT et al. Complete genome sequence of *Corynebacterium pseudotuberculosis* I19, a strain isolated from a cow in Israel with bovine mastitis. *J Bacteriol* 2011;193:323–324.
- Trost E, Ott L, Schneider J, Schröder J, Jaenicke S et al. The complete genome sequence of *Corynebacterium pseudotuberculosis* FRC41 isolated from a 12-year-old girl with necrotizing lymphadenitis reveals insights into gene-regulatory networks contributing to virulence. *BMC Genomics* 2010;11:728.
- D'Afonseca V, Prosdociami F, Dorella FA, Pacheco LG, Moraes PM et al. Survey of genome organization and gene content of *Corynebacterium pseudotuberculosis*. *Microbiol Res* 2010;165:312–320.
- D'Afonseca V, Moraes PM, Dorella FA, Pacheco LG, Meyer R et al. A description of genes of *Corynebacterium pseudotuberculosis* useful in diagnostics and vaccine applications. *Genet Mol Res* 2008;7:252–260.
- Costa MP, McCulloch JA, Almeida SS, Dorella FA, Fonseca CT et al. Molecular characterization of the *Corynebacterium pseudotuberculosis* hsp60-hsp10 operon, and evaluation of the immune response and protective efficacy induced by hsp60 DNA vaccination in mice. *BMC Res Notes* 2011;4:243.
- Selim SA, Ghoneim ME, Mohamed KH. Vaccinal efficacy of genetically inactivated phospholipase D against caseous lymphadenitis in small ruminants. *Int J Microbiol Res* 2010;1:129–136.
- Silva JW, Droppa-Almeida D, Borsuk S, Azevedo V, Portela RW et al. *Corynebacterium pseudotuberculosis* cp09 mutant and cp40 recombinant protein partially protect mice against caseous lymphadenitis. *BMC Vet Res* 2014;10:965.
- Santos AR, Pereira VB, Barbosa E, Baumbach J, Pauling J et al. Mature epitope density – a strategy for target selection based on immunoinformatics and exported prokaryotic proteins. *BMC Genomics* 2013;14:S4.
- Rezende AF, Brum AA, Reis CG, Angelo HR, Leal KS et al. In silico identification of *Corynebacterium pseudotuberculosis* antigenic targets and application in immunodiagnosis. *J Med Microbiol* 2016;65:521–529.
- Mariano DC, Sousa TJ, Pereira FL, Aburjaile F, Barh D et al. Whole-genome optical mapping reveals a mis-assembly between two rRNA operons of *Corynebacterium pseudotuberculosis* strain 1002. *BMC Genomics* 2016;17:315.
- Moura-Costa LF, Bahia RC, Carminati R, Vale VL, Paule BJ et al. Evaluation of the humoral and cellular immune response to different antigens of *Corynebacterium pseudotuberculosis* in Canindé goats and their potential protection against caseous lymphadenitis. *Vet Immunol Immunopathol* 2008;126:131–141.
- Green MR, Sambrook J. *Molecular Cloning – A Laboratory Manual*, 4th ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2012.
- Ramos CR, Abreu PA, Nascimento AL, Ho PL. A high-copy T7 *Escherichia coli* expression vector for the production of

- recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz J Med Biol Res* 2004;37:1103–1109.
27. Forster KM, Hartwig DD, Seixas FK, Bacelo KL, Amaral M et al. A conserved region of leptospiral immunoglobulin-like A and B proteins as a DNA vaccine elicits a prophylactic immune response against leptospirosis. *Clin Vaccine Immunol* 2013;20:725–731.
  28. Fontaine MC, Baird G, Connor KM, Rudge K, Sales J et al. Vaccination confers significant protection of sheep against infection with a virulent United Kingdom strain of *Corynebacterium pseudotuberculosis*. *Vaccine* 2006;24:5986–5996.
  29. Rebouças MF, Loureiro D, Bastos BL, Moura-Costa LF, Hanna SA et al. Development of an indirect ELISA to detect *Corynebacterium pseudotuberculosis* specific antibodies in sheep employing T1 strain culture supernatant as antigen. *Pesquisa Vet Bras* 2013;33:1296–1302.
  30. Vale VL, Silva MC, de Souza AP, Trindade SC, de Moura-Costa LF et al. Humoral and cellular immune responses in mice against secreted and somatic antigens from a *Corynebacterium pseudotuberculosis* attenuated strain: immune response against a *C. pseudotuberculosis* strain. *BMC Vet Res* 2016;12:195.
  31. Jacob E, Hod-Dvorai R, Ben-Mordechai OL, Boyko Y, Avni O et al. Dual function of polycomb group proteins in differentiated murine T helper (CD4<sup>+</sup>) cells. *J Mol Signal* 2011;6:5.
  32. Cardona PJ, Gordillo S, Díaz J, Tapia G, Amat I et al. Widespread bronchogenic dissemination makes DBA/2 mice more susceptible than C57BL/6 mice to experimental aerosol infection with *Mycobacterium tuberculosis*. *Infect Immun* 2003;71:5845–5854.
  33. Matsui H, Yokoyama T, Tanaka C, Sunaga H, Koitabashi N et al. Pressure mediated hypertrophy and mechanical stretch up-regulate expression of the long form of leptin receptor (ob-Rb) in rat cardiac myocytes. *BMC Cell Biol* 2012;13:37.
  34. Heydenreich B, Bellinghausen I, Lund L, Henmar H, Lund G et al. Adjuvant effects of aluminium hydroxide-adsorbed allergens and allergoids - differences *in vivo* and *in vitro*. *Clin Exp Immunol* 2014;176:310–319.
  35. Andreasen LV, Hansen LB, Andersen P, Agger EM, Dietrich J et al. Aluminium hydroxide potentiates a protective Th1 biased immune response against polio virus that allows for dose sparing in mice and rats. *Vaccine* 2015;33:1873–1879.
  36. Abbas A, Lichtman A, Shiv P. *Cellular and Molecular Immunology*, 8th ed. Philadelphia, PA: Elsevier; 2014.
  37. Holstad G, Teige J, Larsen HJ. *Corynebacterium pseudotuberculosis* infection in goats. VIII. the effect of vaccination against experimental infection. *Acta Vet Scand* 1989;30:275–283.
  38. Alharbi KBA. Control of abscess disease of sheep by concurrent vaccination, zinc injection and antiseptic washing. *Res Vet Sci* 2011;4:9–13.
  39. Braga WU. Protection in alpacas against *Corynebacterium pseudotuberculosis* using different bacterial components. *Vet Microbiol* 2007;119:297–303.
  40. Pinho JMR, Dorella FA, Coelho KS, Fonseca CT, Cardoso FC et al. Immunization with recombinant *Corynebacterium pseudotuberculosis* heat-shock protein (Hsp)-60 is able to induce an immune response in mice, but fails to confer protection against infection. *Open Vet Sci J* 2009;3:22–27.
  41. Droppa-Almeida D, Vivas WL, Silva KK, Rezende AF, Simionatto S et al. Recombinant CP40 from *Corynebacterium pseudotuberculosis* confers protection in mice after challenge with a virulent strain. *Vaccine* 2016;34:1091–1096.
  42. Abdulhaqq SA, Weiner DB. DNA vaccines: developing new strategies to enhance immune responses. *Immunol Res* 2008;42:219–232.
  43. Schrijver RS, Langedijk JP, Keil GM, Middel WG, Maris-Veldhuis M et al. Immunization of cattle with a BHV1 vector vaccine or a DNA vaccine both coding for the G protein of BRSV. *Vaccine* 1997;15:1908–1916.
  44. Chaplin PJ, de Rose R, Boyle JS, Mcwaters P, Kelly J et al. Targeting improves the efficacy of a DNA vaccine against *Corynebacterium pseudotuberculosis* in sheep. *Infect Immun* 1999;67:6434–6438.
  45. de Rose R, Tennent J, Mcwaters P, Chaplin PJ, Wood PR et al. Efficacy of DNA vaccination by different routes of immunisation in sheep. *Vet Immunol Immunopathol* 2002;90:55–63.
  46. Brogden KA, Chedid L, Cutlip RC, Lehmkuhl HD, Sacks J et al. Effect of muramyl dipeptide on immunogenicity of *Corynebacterium pseudotuberculosis* whole-cell vaccines in mice and lambs. *Am J Vet Res* 1990;51:200–202.
  47. Cameron CM, Minnaar JL, Engelbrecht MM, Purdom MR. Immune response of merino sheep to inactivated *Corynebacterium pseudotuberculosis* vaccine. *Onderstepoort J Vet Res* 1972;39:24.
  48. Brown CC, Olander HJ, Biberstein EL, Morse SM. Use of a toxoid vaccine to protect goats against intradermal challenge exposure to *Corynebacterium pseudotuberculosis*. *Am J Vet Res* 1986;47:1116–1119.
  49. El-Enbaawy MI, Saad MM, Selim SA. Humoral and cellular immune responses of a murine model against *Corynebacterium pseudotuberculosis* antigens. *Egypt J Immunol* 2005;12:13–19.
  50. Holstad G. *Corynebacterium pseudotuberculosis* infection in goats. IX. The effect of vaccination against natural infection. *Acta Vet Scand* 1989;30:285–293.
  51. Gorman JK, Gabriel M, Maclachlan NJ, Nieto N, Foley J et al. Pilot immunization of mice infected with an equine strain of *Corynebacterium pseudotuberculosis*. *Vet Ther* 2010;11:E1–E8.
  52. Rebouças MF, Portela RW, Lima DD, Loureiro D, Bastos BL et al. *Corynebacterium pseudotuberculosis* secreted antigen-induced specific gamma-interferon production by peripheral blood leukocytes: potential diagnostic marker for caseous lymphadenitis in sheep and goats. *J Vet Diagn Invest* 2011;23:213–220.
  53. Lan DT, Taniguchi S, Makino S, Shirahata T, Nakane A et al. Role of endogenous tumor necrosis factor alpha and gamma interferon in resistance to *Corynebacterium pseudotuberculosis* infection in mice. *Microbiol Immunol* 1998;42:863–870.
  54. de Souza AP, Vale VL, Silva MC, Araújo IB, Trindade SC et al. MAPK involvement in cytokine production in response to *Corynebacterium pseudotuberculosis* infection. *BMC Microbiol* 2014;14:230.

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