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Evaluation of the efficacy of Outer Membrane Protein 31vaccine formulations for protection against *Brucella canis* in BALB/c mice

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Running title: Subcellular vaccines against *Brucella canis* in mice

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Abstract

Canine brucellosis is an infectious disease caused by the Gram negative bacterium *Brucella canis*. Unlike conventional control programs for other species of the genus *Brucella*, currently there is no vaccine available against canine brucellosis and preventive measures are based only in diagnosis and isolation of infected dogs. New approaches are therefore needed to develop an effective and safe immunization strategy against this zoonotic pathogen. In this study, BALB/c mice were subcutaneously immunized with: a) the recombinant (r) *Brucella* Omp31 antigen formulated in different adjuvants (Incomplete Freund Adjuvant, Aluminum Hydroxide, Quil A and Montanide IMS 3012 VGPR), b) the plasmid pCIOmp31 or c) pCIOmp31 plasmid followed by boosting with rOmp31. The immune response and the protective efficacy against *B. canis* infection were characterized. The different strategies induced a strong immunoglobulin G (IgG) response. Besides, spleen cells from rOmp31-immunized mice produced gamma-interferon and IL-4 after *in vitro* stimulation with rOmp31, indicating the induction of a mixed Th1-Th2 response. Recombinant Omp31 administered with different adjuvants as well as the prime-boost strategy conferred protection against *B. canis*. In conclusion, our results suggest that Omp31 could be a useful candidate for the development of a subcellular vaccine against *B. canis* infection.

Key words: *Brucella canis*; Omp31; vaccine; immunogenicity

Introduction

Canine brucellosis, caused by *Brucella canis*, is a worldwide bacterial disease that affects dogs and has been shown to constitute a risk for humans (1). Traditionally the infection has been associated with kennels, but nowadays it has been spread through various dog populations, including shelter and stray dogs (2). It causes mainly reproductive disorders, such as abortions and infertility. Furthermore, signs of canine brucellosis might not become apparent for many years on infected animals (3), making difficult to implement measurements to avoid the spread of the disease to non-infected animals.

Methods to control the disease are based solely in diagnostic tests, such as Rapid Slide Agglutination Test with 2-mercaptoethanol (2ME-RSAT), agar gel immunodiffusion test (AGID) or ELISA (4, 5); and control actions to avoid the contagion of healthy animals (6). While control measures in brucellosis on other animal species include vaccination, at present there is no available vaccine against *B. canis*. On the other hand, despite the continuous development of different serologic techniques, diagnosis remains a complex issue that is not always reliable (7). Moreover, any ideal canine brucellosis control program should rely on a vaccine that contains protective antigens that do not cause misinterpretation of serological results between infected and vaccinated animals.

B. canis, along with *B. ovis*, are the two natural rough species of the genus, a characteristic given by the lack of the O polysaccharide chain of the lipopolysaccharide (8). This particularity becomes relevant since it has been demonstrated that the accessibility of critical outer membrane protein (OMP) epitopes to antibodies has implications in protective immunity, since antibody binding to OMP were demonstrated to be critical for protection against *Brucella* rough species (9-10). Many studies have focused on the OMP properties as immunogens, not only to be used as vaccine candidates but also as diagnostic antigens (11, 12). Experiments on

antibody binding capacity showed that Omp31 (13), along with Omp25 (14) and Omp2b (15) are displayed at high levels and exposed on the outer membrane of *B. canis* and *B. ovis* (16). In spite of the significant variability in the surface phenotype, most of the epitopes of the OMP are conserved among the main pathogenic species of the genus *Brucella* (9, 17). Previous studies demonstrated that a high percentage of *B. canis*-infected dogs developed detectable titers of specific antibodies against rOmp31 from *B. melitensis* (18). Furthermore, the nucleotide sequence of this protein is quite conserved in the genus, and the *B. canis* Omp31 sequence displays only one nucleotide substitution in comparison with *B. melitensis* Omp31 (19). It has also been reported that the administration of a monoclonal antibody against a hydrophilic loop of Omp31 protected against *B. ovis* infection in mice (10, 16). Also, when Omp31 was evaluated as a vaccine candidate it conferred similar protection than *B. melitensis* Rev. 1 against *B. ovis* and *B. melitensis* infection, either as a recombinant protein or as DNA vaccine (pCIOmp31) (20- 21). On the other hand, rOmp31 stimulated a strong cellular and humoral immune response also in rams, which significantly reduced bacterial burden and lesions in organs after *B. ovis* infection (22).

As mentioned, prevention of *B. canis* infection is dependent on sustained screening of dogs. Repeated experience in brucellosis control has shown that the spread of the disease in any animal species can only be prevented or reduced by the use of vaccines (23). Unfortunately, efforts to develop an effective vaccine against *B. canis* in dogs have been unsuccessful to date. Since Carmichael's seminal work in the 1980s, there has been no further research in this matter. In that work, a less mucoid strain (M-) of *B. canis* was used to infect dogs. The results demonstrated that the M- variant met some of the criteria for an immunizing agent (24). Nevertheless, the study failed to provide unequivocal assurance of acceptable attenuation and later communications demonstrated the zoonotic nature of the strain (25, 26).

Subcellular vaccines may represent an alternative, since they can be designed to include only the immunogens required for protective immunity, therefore being safer than whole-inactivated or live-attenuated vaccines (27). Yet, despite these advantages recombinant proteins tend to be poorly immunogenic *in vivo* (28, 29).Thus, the use of potent immunomodulating compounds or suitable delivery systems to stimulate specific strong immune responses is required (30). The appropriate selection of adjuvants is essential in the formulation of novel and efficacious vaccines (31).

We have demonstrated that rOmp31 formulated in Incomplete Freund Adjuvant (IFA) induced protection against *B. ovis* and *B. melitensis* in mice when injected intraperitoneally (20, 21). Both, the use of IFA and the route of immunization are common for experimental immunizations but are not recommended for domestic animals. As we decided to investigate the immunogenicity and the protective capacity of Omp31 against *B. canis* infection in mice, we carefully chose three different safe adjuvants approved for use in dogs: Aluminum hydroxide gel, Quil A saponin and Montanide IMS3012 VGPR (Seppic, France). Also, more appropriate routes of injection were employed. Here, we present the results of this study.

Materials and methods

Animals

BALB/c mice (6 to 8 weeks old) obtained from Universidad de Buenos Aires were acclimated and randomly distributed into experimental groups. Mice were kept in conventional animal facilities with filtered air and handled following international guidelines required for animal experiments under our Faculty Animal Welfare Commission (Acta 087/02, F.C.V., U.N.C.P.B.A, Tandil, Argentina; http://www.vet.unicen.edu.ar).

Bacterial strains

B. canis ATCC RM6/66 and *B. canis* less mucoid strain (M-) were obtained from our *Brucella* culture collection. *B. canis* RM6/66 was used as challenge strain after two serial passages in BALB/c mice and re-isolation from spleens. Bacterial suspension was prepared as previously described (32). Briefly, this strain was grown on Brucella Agar (Britania, Argentina) for 24 h at 37°C. For infection, cells were harvested, spectrophotometrically adjusted in phosphate-buffered saline (PBS) considering an OD_{600} = 0.165 equals to approximately 10^9 colony forming units (CFU)/ml. Exact numbers of cells were assessed retrospectively by dilution and spreading on the required medium (33). Suspension of heat-killed *B. canis* (HKBC) was prepared in the same condition and was inactivated for 1 hour at 80°C.

Antigen production

Recombinant Omp31 (rOmp31) from *B. melitensis* was cloned, expressed in *Escherichia coli* BL21 (DE3) (Stratagene), and purified as previously described (18). Briefly, to purify the soluble protein from the inclusion bodies in urea solution, a Niquel chelated resin (HisLinkTM, Promega) was used following the manufacturer´s instructions, in batch format and denaturing conditions. The presence and purity of rOmp31 in eluates was checked by SDS-PAGE and Coomassie blue staining. Eluates containing the purified protein were dialyzed overnight against deionized water with 1 mM phenylmethylsulfonyl fluoride (PMSF) and stored at -70°C. Protein concentration was determined by the Bicinchoninic Acid Assay (BCA) with bovine serum albumin as standard (Pierce, Rockford, IL).

DNA vaccine coding for Omp31 was expressed and purified as previously described (34). *E. coli* JM109 cells were transformed with pCI-neo vector (Promega, Madison, WI) containing the gene of the Omp31. The plasmid was amplified and isolated using “megaprep” plasmid

isolation columns (Genelute, Sigma). Purity and concentration of DNA was determined by spectrophotometry at 260/280 nm.

Adjuvants and preparation of the immunogens

Aluminum hydroxide (AH) gel was prepared as described previously (35). To adsorb the antigen, the aluminum hydroxide suspension was mixed with equal volume of rOmp31 in PBS and incubated for 30 min at room temperature. The AH-adsorbed rOmp31 antigen was washed, and the final pellet was resuspended in PBS. Incomplete Freund Adjuvant (IFA) was prepared mixing Marcol 52 (kindly provided by Biogenesis, Argentina) with 10% of Arlacel (Sigma, St. Louis, MO, USA) in order to facilitate emulsification with the immunogen. Montanide IMS 3012 VGPR (MON) (Seppic, France) and QUIL A (Brenntag Biosector, Denmark) were used according to the manufacturer's instructions.

Immunizations and experimental design

Mice were randomly separated into groups (n=10). Each group received different antigens according to the vaccination schedule. Mice immunized with pCIOmp31 were injected three times (days 0, 15 and 30) by intramuscular (i.m.) route (100 µg in 100 µL of PBS). Mice of prime-boost group (pCIOmp31 + boost) was immunized with the same plasmid schedule followed by a final subcutaneous (s.c.) booster (fourth injection) performed with rOmp31-IFA formulation (30 µg in 200 µL). Recombinant Omp31 formulated in the different adjuvants were administered two times (days 30 and 45) by s.c. route (30 µg in 200 µL).

As a positive control vaccine, HKBC *B. canis* emulsified in IFA (1 x 10^9 CFU in IFA) was administered twice subcutaneously (days 30 and 45) according with our previous work (28).

157 In addition, a PBS injected group was also included (negative control). All schedules were
158 synchronized in order to inject simultaneously the last boost in all groups.

159 Animals were controlled by a veterinarian to evaluate general status and local adverse
160 reactions in the site of injection.

161

162 *Indirect ELISA assays*

163 Mice were bled by submandibular puncture every 2 weeks before and after the challenge.
164 Serum reactivity to rOmp31 was determined by indirect ELISA. The plates were sensitized with
165 0.1 µg of rOmp31 in 100 µL of PBS pH 7.2 at 4°C overnight. Blocking was done with PBS plus
166 0.05% Tween 20 and 3% skim milk. Mice sera were diluted 1/100 in PBS plus 0.05% Tween 20
167 and 1% skim milk and were incubated for 1 h at 37°C. Bound antibodies were detected by a goat
168 anti-mouse IgG (whole-molecule) conjugated to horseradish peroxidase (Sigma, Germany)
169 diluted in the same buffer. The reaction was developed by adding 2,2'-azino-bis(3-
170 athylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma, Germany) 1mM in
171 citrate-phosphate buffer containing 0.03% H_2O_2. The absorbance was determined using a
172 microplate reader (Multiskan EX, Labsystem). The cutoff value for the assay was calculated as
173 the mean of the specific optical density plus 3 standard deviations (SD) for 20 sera obtained from
174 non-immunized mice and assayed at dilutions of 1:100. The titer of each serum was calculated as
175 the last serum dilution yielding a specific optical density higher than the cutoff value.

176

177 *Cytokine production*

178 To evaluate and characterize the cellular immune response induced by the immunization
179 strategies, 5 mice per group were sacrificed 30 days after the last immunization. The spleens were

aseptically removed and homogenized in RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine, 100 U of penicillin per mL, 50 µg of streptomycin per mL, and 10% fetal calf serum. Cells were cultured at 4 x 10^6/mL in duplicate wells with Omp31 (5 µg/mL), concanavalin A (ConA; 2.5 µg/mL) (Sigma) or with culture medium alone. Cell cultures were incubated for a period of 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air. At the end of the incubation, cell culture supernatants were collected, aliquoted, and frozen at -70°C until analyzed for gamma interferon (IFN-γ) and interleukin-4 (IL-4) production by sandwich ELISA using paired cytokine-specific monoclonal antibodies according to the manufacturer's instructions (Pharmingen, San Diego, California).

Protection experiments

Thirty days after the last immunization 5 mice per group were challenged by i.p. inoculation with 5.5 × 10^5 CFU *B. canis* RM6/66 in 200 µL of PBS. Mice were sacrificed by cervical dislocation 30 days after being challenged, and their spleens were removed aseptically, weighed and kept at -20°C until processed. To determine the infection level, spleens were thawed and individually homogenized using an appropriate volume of PBS in sterile plastic bags, serially diluted (ten-fold) and each dilution was seeded onto two plates of TSAYE medium. After 4 days of incubation, CFU were counted and expressed by the $\log_{10}$ per spleen value as previously described (32, 33).

Statistical analysis of data

The CFU data were normalized by log transformation and evaluated by ANOVA followed by Dunnett's *post hoc* test. The Kruskal-Wallis test and ANOVA were used to compare antibody

and cellular responses, respectively. Graphs were performed using Graph Pad software, version 4.0, San Diego, CA.

Results

Prime-boost strategy and recombinant Omp31-based vaccines developed significant specific IgG responses.

To evaluate the humoral immune response elicited by the different strategies of immunization, anti-Omp31 IgG antibodies were measured by specific indirect ELISA in sera from immunized and control mice. Sera from mice injected with PBS and heat-killed *B. canis* (HKBC) which served as controls for the protection experiments were included. pCIOmp31 + boost strategy, rOmp31-AH gel, rOmp31-IFA or rOmp31-Quil A formulations elicited a strong specific IgG response after the second boost ($P<0.01$) (**Figure 1**). In contrast, pCIOmp31, rOmp31-Montanide and HKBC induced a weak humoral immune responses against rOmp31 ($P>0.05$). Thirty days after the i.p. challenge with *B. canis* RM6/66, specific anti-Omp31 antibody levels increased significantly in groups immunized with plasmid vaccine, pCIOmp31 + boost or Omp31-Quil A (**Figure 1**). On the contrary, *B. canis* challenge was unable to boost the response of mice immunized with rOmp31-HA or rOmp31-IFA. Neither the animals injected with PBS nor the HKBC-immunized animals showed anti-Omp31 antibodies. These results are consistent with our previous reports in which we tested different Omp31 strategies against another rough species of the genus such as *B. ovis* (21, 34). Anyway, antibody response against *B. canis* antigens other than Omp31 was observed in all groups after challenge, as indicated by RSAT positive results (not shown).

Recombinant Omp31-based vaccines induced specific cellular immune responses.

In order to obtain further information on the type of immune response induced by the different immunization protocols at the time of bacterial challenge, we used ELISA to investigate cytokine secretion in rOmp31-stimulated spleen cell cultures from the different immunization groups. Recombinant Omp31 significantly stimulated the production of IFN-γ and IL-4 in splenocytes from mice immunized with rOmp31 formulated in the different adjuvants and from pCIOmp31 + boost-vaccinated and HKBC-immunized mice ($P<0.01$). On the contrary and as reported (21), pCIOmp31 immunization did not induce IFN-γ and IL-4 production. Splenocytes from mice immunized by pCIOmp31 + boost, rOmp31-IFA and HKBC produced significantly ($P<0.01$) higher levels of IFN-γ than cells from mice given rOmp31-AH, rOmp31-MON or rOmp31-QUIL A ($P<0.05$). Also, significant higher levels of IL-4 were detected in groups immunized with rOmp31-HA and HKBC ($P<0.01$). In contrast, specific secretion of IL-4 was comparable between the other groups of immunized mice (**Figure 2**). Cells from PBS-immunized mice did not secrete IFN-γ or IL-4 when stimulated with rOmp31. Spleen cells from all immunized mice produced both cytokines in response to Con A with no significant differences observed among the groups. These results indicate that rOmp31 in different adjuvants injected subcutaneously induced a mixed Th1–Th2 cytokine response.

The different recombinant Omp31-based strategies protect BALB/c mice against *B. canis infection*

Thirty days after the last immunization, mice were challenged by an i.p. injection of 5.65 x 10^5 UFC of *B. canis* RM6/66. Thirty days later, mice were sacrificed and their spleens removed and processed for bacterial burden. *B. canis* growth was significantly inhibited ($P<0.05$) in

groups immunized with rOmp31 with every adjuvant and the pCIOmp31 + boost strategy in comparison with PBS control (**Table 1**). pCIOmp31 was the only vaccine formulation that failed to give any level of protection against *B. canis* infection. As previously reported by our group when using heat-killed whole bacterial cells (21, 34), the control vaccine HKBC in IFA induced the highest protection level (3.48 log of protection).

All mice immunized with rOmp31 or HKBC emulsified in IFA developed large non-septic abscesses in the site of injection. This lesion persisted several weeks and mice exhibited also local hair loss. None of the other strategies induced local or systemic adverse reactions (not shown).

Discussion

Traditional approaches to *Brucella* vaccine development employs whole cell vaccines which are composed of suspensions of whole killed or attenuated cells (36). Nowadays, approved vaccines for use in ruminants for preventing brucellosis are based on attenuated strains (37). While these vaccines have reduced virulence for animals, they are pathogenic for humans and they are resistant to antibiotics used in the treat of human brucellosis (36). Therefore, these vaccines have a restricted use in animals because they can induce abortion in pregnant females (36). In view of these risks, many researchers have investigated alternative vaccination strategies for brucellosis, including the use of subunit vaccines based on recombinant proteins or DNA (27). Alternatively, the use of adjuvants in combination with antigens might be an alternative to enhance vaccination efficacy. Owing to the lack of suitable strategies to protect animals and humans against canine brucellosis, our goal is to explore different approaches to develop and test an appropriate vaccine against *B. canis*.

Outer membrane proteins of *Brucella* spp. have been characterized and studied as potential immunogenic or protective antigens (10, 16). In particular, recombinant Omp31-based vaccines (20, 21, 22), alone or associated with rough lipopolysaccharide conferred protection against *B. ovis* in mice (33) and rams (22). These results were encouraging for the testing of Omp31-delivery strategies against *B. canis* in mice.

B. canis, as any other *Brucella* spp., is a facultative intracellular pathogen. Cell-mediated immunity plays a critical role in protection against virulent *Brucella* infection. However, previous studies have shown that specific antibodies bind to OMPs of rough *Brucella* microorganisms (10). Moreover, it has been shown that antibodies against Omp31 can mediate complement-dependent bacteriolysis of *B. ovis* (22). *In vivo* this lytic mechanism could have a protective role during the bacteriemic phase of *B. ovis* or *B. canis* infections before the entry of bacterium to their target cells. In this work, all rOmp31 administered with different adjuvants induced a vigorous IgG response as well as IL-4 and gamma IFN suggesting the induction of a mixed Th2/Th1 immune response (20, 34). We speculate that differences in the magnitude of the immune response could be associated with the adjuvant and/or the administration route used. Furthermore, the coordinated immune response against rOmp31 conferred protection against *B. canis* infection in mice independently of the adjuvant formulation used. Levels of protection were in the range of the ones obtained using Omp31 with the other rough strain of the genus (*B. ovis*) in the mouse model (20, 21, 34). However, the protection afforded was always significantly lower than the one provided by immunization with HKBC (control vaccine). In our experience, this is always the case when using whole death cells or attenuated vaccines comprising the whole antigenic load of a microorganism (20, 31, 38). Anyway, most of these preparations interfere with diagnosis (37, 38). While protection afforded could be improved using a multiple subunit vaccine, it also remains possible that a more effective antigen or a better adjuvant might lead to a

298 higher degree of protection with a monovalent subunit vaccine. Previously, we have
299 demonstrated that the chimerical protein based on the addition to the N-termini of BLS of a 27-
300 mer peptide containing the exposed loop epitope of Omp31 (BLSOmp31) is able to develop
301 strong humoral and cellular responses and confers protection against *B. canis* in mice (38).

302 When selecting immunization strategies for a trial with pets, the site of injection and the
303 adjuvant to be used should be considered. Vaccines containing recombinant antigens may be less
304 reactogenic but also less immunogenic, thus necessitating the inclusion of an adjuvant (28).
305 However, the adjuvant should be chosen considering the benefits and risks for the target species.
306 In this study, we selected three commercial adjuvants approved for use in dogs, along with IFA,
307 since it has been used in previous works of Omp31 (20, 21, 34). In addition, the subcutaneous
308 route was chosen as a common route for vaccine administration in dogs. As expected, the severity
309 of local reaction occurring after IFA emulsified vaccines in mice could rule out this adjuvant for
310 future trials in dogs. Nevertheless, Omp31 formulated in the other adjuvants induced statistically
311 similar levels of protection, which reinforces the potentiality of this immunogen to become an
312 effective vaccine against *B. canis* in the susceptible host.

313 In conclusion, recombinant Omp31 could be a useful candidate for the development of a
314 subunit vaccine against *B. canis* since it elicits antigen-specific humoral and cellular responses
315 and conferred protection in the mouse model.

316

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Legends

Figure 1. Antibody response against recombinant Omp31 in mice immunized with different strategies. Mice were immunized as described in Material and Methods. IgG specific antibodies against rOmp31 were evaluated by indirect ELISA preinoculation (30 days after last immunization) and postinoculation (30 days after challenge with *B. canis* RM6/66). Each symbol represents the mean ± S.D. of ten and five mice, respectively. The figure shows a representative experiment from two performed with similar results (** $p< 0.01$).

Figure 2. Determination of A) IFN- γ and B) IL-4 levels in supernatant of spleen cells culture from mice immunized with different strategies. The graph shows the mean ± S.D. of cells producing IFN-γ after stimulation with rOmp31 as described in Materials and Methods. Spleen cells (4×10^6/mL) were stimulated with complete medium RPMI 1640 or rOmp31 (5 μg/mL) for 48 h. Levels of IFN- γ (upper panel), IL-4 (lower panel) in the cell supernatants were quantified (pg/mL) by MAb-capture ELISA. Each bar represents the mean of duplicates ± S.D. of the response of spleen cells from five individual mice (* $p< 0.05$ and ** $p< 0.01$).

Table 1. Protection against *B. canis* in mice immunized with Omp31 using different strategies of immunization

Figure 1

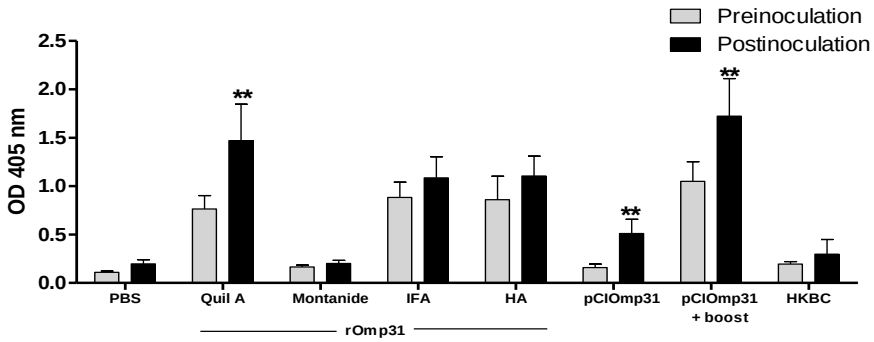
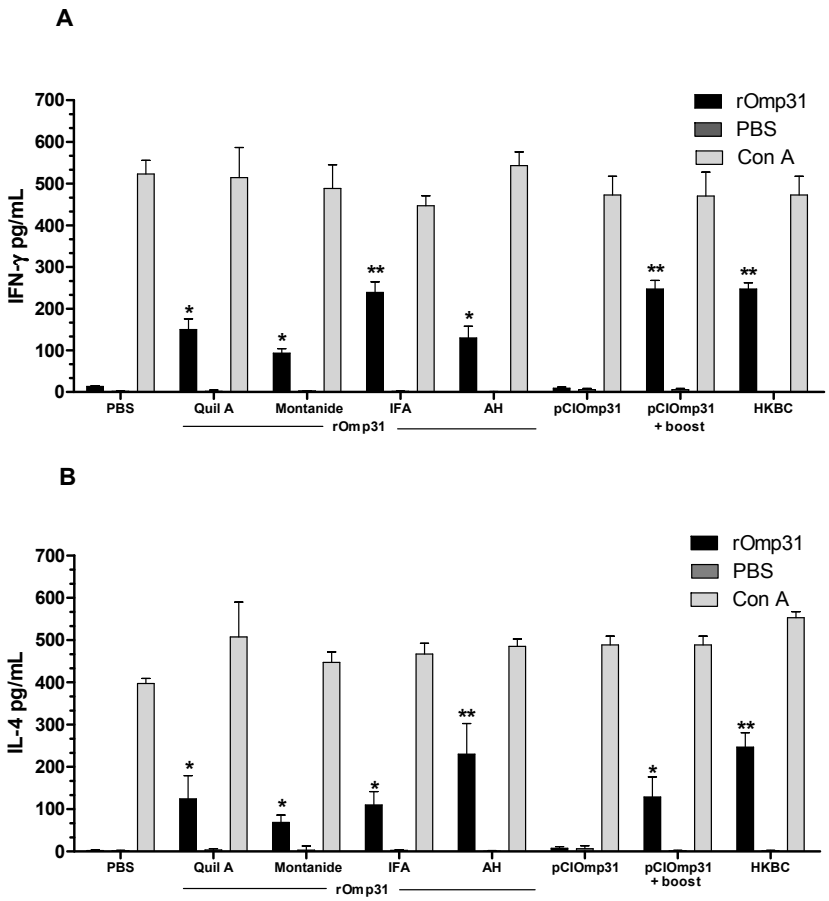


Figure 2



1 **Table 1**.

Vaccine (n=5)	Adjuvant	Log_{10} *B. canis* at spleen[a]	Log units of protection
PBS	-	6.18 ± 0.11	-
rOmp31	Quil A	4.14 ± 0.68	1.86 *
rOmp31	Montanide	4.63 ± 0.50	1.42 *
rOmp31	IFA	4.37 ± 0.36	1.66 *
rOmp31	HA	4.37 ± 0.82	1.65 *
pCIOmp31	-	5.67 ± 0.66	0.66
pCIOmp31+ boost	IFA	4.53 ± 0.92	1.50 *
HKBC	IFA	2.25 ± 0.58	3.48 **

[a]The content of bacteria in spleens is represented as the mean log CFU ± SD per group.

*Significantly different from PBS –immunized mice $p<0.05$ estimated by Dunnett´s t-test.

**Significantly different from PBS –immunized mice $p<0.01$ estimated by Dunnett´s *t*-test.