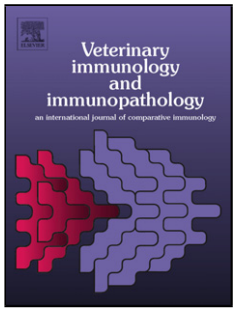


Title: Polymeric antigen BLSOmp31 in aluminium hydroxide induces serum bactericidal and opsonic antibodies against *Brucella canis* in dogs

Author: Maria Clausse Alejandra G. Díaz Romina P. Pardo Vanesa Zylberman Fernando A. Goldbaum Silvia M. Estein

PII: S0165-2427(16)30325-7
DOI: http://dx.doi.org/doi:10.1016/j.vetimm.2016.11.004
Reference: VETIMM 9581

To appear in: *VETIMM*

Received date: 13-5-2016
Revised date: 15-11-2016
Accepted date: 28-11-2016

Please cite this article as: Clausse, Maria, Díaz, Alejandra G., Pardo, Romina P., Zylberman, Vanesa, Goldbaum, Fernando A., Estein, Silvia M., Polymeric antigen BLSOmp31 in aluminium hydroxide induces serum bactericidal and opsonic antibodies against Brucella canis in dogs.Veterinary Immunology and Immunopathology http://dx.doi.org/10.1016/j.vetimm.2016.11.004

Polymeric antigen BLSOmp31 in aluminium hydroxide induces serum bactericidal and opsonic antibodies against *Brucella canis* in dogs

Maria Clausse[a,d*]; Alejandra G. Díaz[a,d]; Romina P. Pardo[b]; Vanesa Zylberman[b,c,d]; Fernando A. Goldbaum[b,c,d]; Silvia M. Estein[a,d]

[a] Laboratorio de Inmunología, Departamento de Sanidad Animal y Medicina Preventiva (SAMP), Centro de Investigación Veterinaria de Tandil (CIVETAN-CONICET-CIC), Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina.

[b] Inmunova S.A.

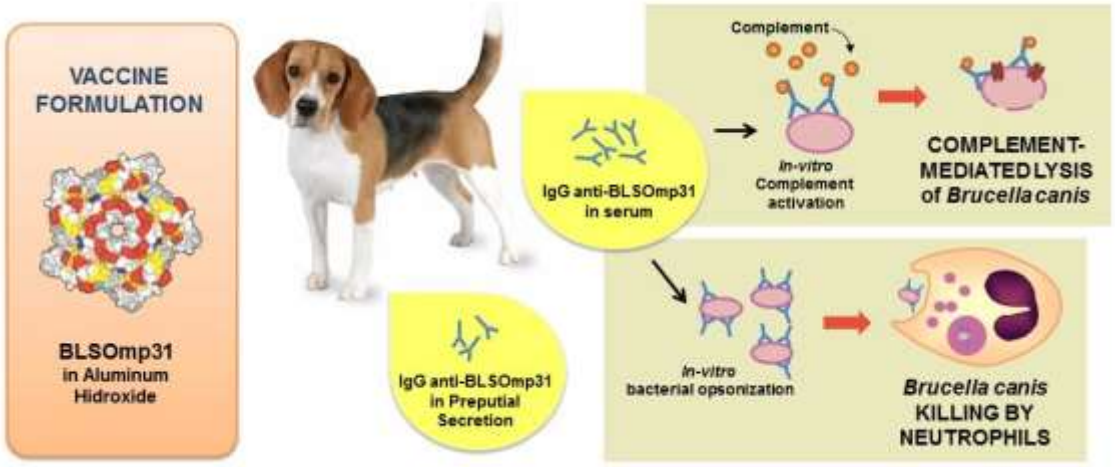
[c] Fundación Instituto Leloir e Instituto de Investigaciones Biológicas Buenos Aires-CONICET.

[d] Consejo Nacional de Investigaciones Científicas (CONICET)

*Corresponding author. Mailing address: Laboratorio de Inmunología, CIVETAN, CONICET, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Buenos Aires, Argentina. Pinto 399. Buenos Aires, Argentina. TEL-FAX: (54-11) 22494439850.

Email: mclausse@vet.unicen.edu.ar

Graphical abstract



Highlights

- BLSOmp31 in aluminum hydroxide elicited a strong humoral immune response in dogs
- This vaccine induced specific IgG in preputial secretions in dogs
- Specific antibodies stimulated uptake and killing of *Brucella canis* by neutrophils
- Antibodies anti-BLSOmp31 showed a high complement-dependent bactericidal activity

Abstract

Polymeric antigen BLSOmp31 is an immunogenic vaccine candidate that confers protection against *Brucella canis* in mice. In this preliminary study, the immunogenicity and safety of BLSOmp31 adsorbed to aluminum hydroxide gel (BLSOmp31-AH) were evaluated in Beagle dogs. In addition, the potential to elicit serum antibodies with

complement-dependent bactericidal activity and/or to enhance phagocytosis by neutrophils were analyzed. Dogs were immunized three times with BLSOmp31-AH by subcutaneous route, followed by an annual booster. The vaccine elicited specific antibodies 3 weeks after the first immunization. Annual booster induced comparable antibody response as the primary series. Humoral immune response stimulated by BLSOmp31-AH did not interfere with routine agglutination test for canine brucellosis. Antibodies demonstrated a high complement-dependent bactericidal activity against *B. canis*. Moreover, opsonization by immune serum not only stimulated binding and uptake of the bacteria by neutrophils but effectively enhanced the destruction of *B. canis*. Specific IgG was detected in 3/4 immunized dogs in preputial secretions. The antibody profile corresponded to a marked Th2 response, since IgG1 prevailed over IgG2 and cellular immune response was not detected *in vitro* or *in vivo*. These results require further evaluation in larger field studies to establish the full prophylactic activity of BLSOmp31 against canine brucellosis.

Keywords: BLSOmp31, aluminum hydroxide, canine brucellosis, dog, opsonic antibodies, bacteriolytic antibodies

Introduction

Canine brucellosis is caused by *Brucella canis* and constitutes the main reproductive primary infection in dogs. The organism affects the reproductive system, causes abortion and subsequent delayed conception and may even lead to permanent infertility (Wanke, 2004). This species was historically considered far less pathogenic to human than other

Brucella spp, although throughout the last two decades *B. canis* infection has become an emerging concern to public health (Lucero et al., 2010; Marzetti et al., 2013). The disease, which had been related to kennels and breeders, appears now as an endemic disease among stray dog populations in developing countries (Lucero et al., 2010; Marzetti et al., 2013). Nevertheless, the most devastating impact of canine brucellosis is the one affecting kennels, where it causes high costs in terms of lost litters and breeding stock, as well as veterinary and diagnostic costs and negative reputation of the kennel (Bosseray and Plommet, 1990).

Many efforts have been made to produce vaccines against *Brucella* spp. that affect farm animals, although this matter has not been studied in dogs. It is reasonable to expect that an effective vaccine would represent a major advance on the control of this disease, providing a useful tool to reduce the spread of the infection not only in kennels but also among stray dogs and pets.

Traditional vaccines against smooth *Brucella*, licenced for use in cattle, sheep or swine, are attenuated strains (Bosseray and Plommet, 1990). Currently, research on canine brucellosis vaccines is being conducted employing different techniques and research strategies. A non-polar virB10 mutant and a virB11 deletion mutant were constructed in *B. canis*, which elicited a protective immunity in mice (Palomares-Resendiz et al., 2012) but vaccination with whole-cell vaccines would interfere with current diagnostic test.

DNA vaccines codifying for eight different proteins against *B. canis* infection in BALB/c mice have also been studied (Lee et al., 2011). The failure to demonstrate significant protection of these vaccines was probably due to excessive challenge dose used (2×10^8 Colony Forming Unit (CFU)/dose) compared to the dose that had been reported (Clausse and Estein, 2011) and used in-protection tests by our group (Clausse et al., 2013; Clausse et al., 2014). Recently, two VirB proteins were able to induce a significant humoral

and cellular immune responses, but protection tests against *B. canis* challenge have not been undertaken (Pollak et al., 2015).

Among the antigens evaluated by our research group, the outer membrane protein of 31-34 kDa (Omp31) from *B. melitensis* confers protection against *B. canis* (Clausse et al., 2014). However, our leading vaccine candidate is the recombinant polymeric antigen BLSOmp31, which comprises an immunologically active domain of Omp31 covalently bonded to the N-termini of *Brucella* Lumazine Synthase (BLS) cytosolic protein. BLS is a remarkably stable decameric protein with the capability for presentation of heterologous peptides to the immune system in a highly ordered three-dimensional array and behaves as a potent adjuvant when injected as a protein or as a DNA vaccine (Laplagne et al., 2004; Velikovsky et al., 2003; Zylberman et al., 2004). The recombinant BLSOmp31 protein formulated in different adjuvants conferred remarkably high levels of protection against *B. canis* challenge in BALBc mice (Clausse et al., 2013) and also conferred protection in mice and rams against *B. ovis* (the other naturally rough strain of the genus) (Cassataro et al., 2007; Estein et al., 2009). The immunization strategies induced a mixed Th1/Th2 response and high specific IgG titers which did not interfere in current serological test (RSAT: Rapid Slide Agglutination Test) for diagnosis of canine brucellosis (Clausse et al., 2013).

In the present study, we evaluate the immunogenicity and safety of BLSOmp31 adsorbed to aluminum hydroxide gel (BLSOmp31-AH), taking into account its wide acceptance and extensive use in companion animal vaccines (HogenEsch, 2002). Systemic and mucosal humoral immune responses, bactericidal activity mediated by antibodies and cellular immune response were investigated throughout a two-year trial.

Materials and methods

1. Dogs

Five Beagle male 5-month-old dogs seronegative to RSAT test were used and were treated following the guidelines for animal experimentation of the USA National Institute of Health. This work was approved by the Ethical Committee of Animal Research of Facultad de Ciencias Veterinarias, UNCPBA, Argentina (Acta 087/02).

Dogs received routine vaccinations against parvovirosis, distemper, hepatitis, parainfluenza, coronavirosis and kennel cough.

2. Vaccine formulation

Recombinant BLSOmp31 was expressed in *Escherichia coli* BL21(DE3) (Stratagene, La Jolla, CA) and was purified by fast-protein liquid chromatography as previously described (Cassataro et al., 2007). AH gel was prepared as described previously (Margni, 1996). To adsorb the antigen, AH gel was incubated with BLSOmp31 in phosphate-buffered saline (PBS) for 30 minutes at room temperature with agitation. After one wash, loaded gel was suspended and diluted by 20% in PBS.

3. Immunization schedule

Four dogs were injected subcutaneously with 200 μg of BLSOmp31-AH vaccine in the same anatomic region (dorsal of right thoracic wall). One animal remained unvaccinated as negative control. Dogs received 3 booster shots spaced 3 weeks. In addition, an annual booster was performed. Standard health parameters of immunized dogs were recorded by a veterinarian.

4. Sample collection

Blood and secretion samples were collected from dogs prior to primary immunization and then every 2-4 weeks. Samples of blood were taken into sterile tubes (BD Vacutainer) with or without heparin by jugular venipuncture. Saliva, preputial and lacrimal secretions were collected with cotton swabs, placed in PBS added with 0.1% Sodium Azide and incubated overnight at 4ºC. Samples were centrifuged at 6000 rpm at 4ºC for 10 min and stored at -80ºC.

5. Analysis of humoral immune response

5.1 Determination of specific IgG and IgA levels in serum and secretions

Serum reactivity against BLSOmp31 was determined by indirect ELISA. Briefly, 96 well plates (NUNC, Maxisorp, Denmark) were sensitized with BLSOmp31 (0.3 μg/well) in PBS pH 7.2. Blocking was done with PBS plus 0.05% Tween 20 and 3% skim milk (San Regim, Argentina). To determine IgG, dog sera and secretions were diluted in the blocking solution 1/200 and 1/5, respectively. Secretions were diluted 1/2 to determine IgA. Bound IgG antibodies were detected by Protein A conjugated to horseradish peroxidase (Sigma, USA) diluted 1/5000 for serum and 1/2000 for secretions. For IgA determination in secretions, goat anti-dog IgA (1/1000) followed by anti-goat IgG (1/1000) were used. All incubations were done incubated in agitation at 37°C for 1 hour. Enzyme activity was revealed with 1 mM 2,2′-azino-di (3-ethylbenzothiazoline-sulphonic acid) (ABTS) and absorbance was read at 405 nm in automatic ELISA reader (Multiskan EX, Labsystems). Antibody levels were expressed as the arithmetic mean ± SEM of the O.D. obtained from the duplicates for each dog.

Antibody sub-isotyping of sera of immunized dogs was done to characterize the Th1/Th2 profile of the elicited response (Carson et al., 2009). The conditions were the same as for isotype determination, except that goat anti-dog IgG1 and sheep anti-dog IgG2 antibodies (Sigma) were detected by incubation with anti-goat IgG or anti-sheep IgG–horseradish peroxidase conjugates, respectively. The cutoff value for the assay was calculated as the mean specific O.D. plus 3 standard deviations (S.D.) for 20 sera from non-immunized dogs.

5.2. Rapid Slide Agglutination Test

Serum samples were assayed in RSAT test. The antigen was prepared at Laboratorio de Inmunología, Facultad de Ciencias Veterinarias (UNCPBA) using the strain (M−) variant of *B. canis* (Clausse and Estein, 2011). Serum and RSAT antigen were mixed on a glass slide and reaction was read after 1–2 minutes.

5.3. Complement-mediated bacteriolysis assay

Sera collected at week 10 were tested for the ability to promote *in vitro* complement-mediated killing of *B. canis*. This assay was performed in 96 flat-bottomed polystyrene well plates as previously described for *B. ovis* (Estein et al., 2009). Briefly, 1.0×10^4CFU of *B. canis* RM6/66/mL (50 μL) were suspended in PBS containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ and incubated with the dog heat inactivated serum samples (50 μL) for 90 min at 37°C. As source of complement, 40 μL of fresh serum from a seronegative dog was dispensed in appropriate wells. Controls, in duplicate, included antibody–*Brucella* mixtures with (i) both heat inactivated complement sources and (ii) without complement. Also, sera from a *B. canis* infected dog (RSAT-positive) and sera from a non-infected dog were used as positive and negative controls, respectively. An aliquot of each well was plated in

duplicate on required medium. The mean percentage of bacterial killing (%K) was calculated as: %K = 100 × 1 − (mean number of CFU mL−1 after incubation/mean number CFU mL−1 before incubation).

5.4. Serum activity in an opsonocytophagic and killing neutrophil assay

Neutrophils were prepared from blood collected from a seronegative healthy adult Beagle. Blood was mixed with half a volume of 6% dextran in PBS and incubated at room temperature for 1 h. The leucocytes were pelleted by centrifugation, and remaining erythrocytes were lysed with hypotonic solution. Separation from mononuclear lymphocytes was done by centrifugation over Ficoll-Paque Plus (GE Healthcare) at 400 *x g* for 35 minutes. Neutrophils were washed twice in PBS and suspended in Hanks Buffer Saline Solution (HBSS) at 1 x 10^6 neutrophils/mL. Cell viability was >98%, as determined by Trypan Blue exclusion. The purity of the final neutrophil preparation was checked with Giemsa staining.

B. canis RM6/66 was grown overnight in Brucella agar, suspended in HBSS and photometrically adjusted to an OD_{600} of 0.165 (1 x 10^9 CFU/mL) and diluted 1:100 in HBSS. Serum from (i) vaccinated dogs, (ii) a seronegative dog and (iii) an infected dog (RSAT positive) were filtered and inactivated for 30 minutes at 56°C. Aliquots of bacterial suspension were then incubated with 10% of sera for 30 minutes at 37°C in agitation. A control suspension was incubated with PBS alone.

The phagocytic killing assay was performed by mixing equal parts of the neutrophil and *B. canis* suspensions. The reaction mixture was incubated at 37°C with agitation. One sample was taken at different times (0, 20 and 60 minutes) and centrifuged to separate leucocytes from bacterial cells (100 *x g*, 10 minutes, 4°C). Supernatant was removed carefully, washed

3 times with ice cold PBS, suspended in ice cold deionized water and vortexed to lyse the cells and release intracellular bacteria. Intracellular and extracellular bacterial suspensions were diluted and plated onto Brucella agar plates (quadruplicates), and incubated at 37°C for 72 h to determine bacterial count. Controls included neutrophils with bacteria incubated with seronegative or RSAT positive serum. At the concentrations of antisera used in the opsonic killing assay, there was no reduction or increase in CFU in samples lacking phagocytes.

The percentage of killing was calculated by determining the ratio of the number of CFU surviving in intracellular or extracellular compartments to the number of CFU originally added, determined by retrospective colony count (Hampton et al., 1994).

6. Specific cell mediated immune response evaluation

Cell mediated immune response was assessed *in vitro* by measurement of IFN-γ levels expressed by stimulated lymphocytes in whole blood assays (Carson et al., 2009). Blood collected at week 13 was incubated for 48 h with BLSOmp31 (20 μg/well) for 3 days at 37°C in a 5% CO_2 atmosphere. Control cultures were incubated with Pokeweed mitogen (SIGMA) (10 μg/well) or PBS. Supernatants were stored at −80 °C. Measurement of IFN-γ levels was carried out by quantitative ELISA using DuoSet kits (R&D Systems, UK).

In order to evaluate *in vivo* cellular immune response, thirteen weeks after first immunization dogs were tested for delayed-type hypersensitivity reaction to BLSOmp31 by intradermal injection of 15 μg in 50 μL of apyrogenic saline solution in the abdominal wall. The diameter of induration was measured 24, 48 and 72 h later.

7. Statistical analysis

Data from specific IgG ELISA, bacteriolysis, and phagocytic-killing assays were analyzed by ANOVA followed by Dunnett´ *post hoc* test. Differences between subisotypes IgG1-IgG2 were analyzed by Student's t test. The analysis and graphs were performed using Graph Pad software (version 4.0, San Diego, USA).

Results and Discussion

In this work, we evaluated BLSOmp31 in AH as a vaccine candidate against canine brucellosis (Clausse et al., 2013). BLSOmp31-AH vaccine was well tolerated without systemic or local adverse events. Specific anti-BLSOmp31 serum IgG antibodies were strongly induced after prime vaccination in all dogs and remained stable up to week 13, with significant difference from the non immunized dog ($p<0.001$) (Figure 1A). Then, IgG levels declined, with some individual differences. At week 45, only one immunized dog did not show specific IgG. After boost, IgG antibody titers reached original levels and decreased with kinetics very similar to the first immunizations. These results suggest that this vaccine would probably require an annual or bi-annual vaccination to boost the memory cells as contemplated for other recombinant antigens (Welborn et al., 2011). Additionally, antibodies stimulated by BLSOmp31-AH did not interfere with routine agglutination test (RSAT) throughout the experiment, so this vaccine would be compatible with the current serology-based control of the disease.

BLSOmp31 induced a marked Th2 immune response in which IgG1 prevailed over IgG2 (Figure 2) reflecting the role of Th2 cytokines in class switching by B cells. This, together with the lack of specific cellular immune response *in vitro* (Figure 2), confirms that AH induced a Th2 polarized immune response. Intradermal reaction also resulted

negative (data not shown). This observation differs from the profile developed in the mouse model by the same formulation, where a more balanced Th2/Th1 response was observed (Laplagne et al., 2004). Therefore, the mouse model could be unsuccessful in predicting dog immune response.

In order to evaluate whether immunization with BLSOmp31-AH induced a mucosal immune response, specific IgA and IgG antibodies in saliva, preputial and lacrimal secretions were assessed (Figure 1B). Specific IgG titers were only detected in preputial secretions of 3/4 immunized dogs, with great variability between individuals. However, IgG levels correlate with a stronger and sustained humoral response in serum. These antibodies could prevent bacterial colonization and infection when *B. canis* enters by venereal route. Recently, studies carried out by our group showed that intranasal administration of BLSOmp31 loaded in chitosan microspheres was able to induce systemic and mucosal immune responses in sheep (Díaz et al., 2016). Our results encourage us to search for an adequate route of immunization and BLSOmp31 delivery system to improve the mucosal immune response in dogs.

A good understanding of immunity generated against pathogens is important for developing an effective vaccine. We previously reported that sheep anti-BLSOmp31 antibodies showed bactericidal activity against *B. ovis* in the presence of an homologous source of complement (Estein et al., 2009). In this work, sera collected at week 10 after first immunization showed significant bactericidal activity against *B. canis* ($p<0.001$), although lower than positive control serum (infected dog) (Figure 3). Complement inactivation inhibited the lysis, indicating that this component was essential for the lytic activity (data not shown). BLSOmp31 antibodies were not efficacious unless in combination with a

complement indicating that bactericidal activity was due to lytic complement and not to other putative antibody-mediated effect.

Different studies have demonstrated that *Brucella* stimulates a low activation of phagocytic cells in comparison to other Gram negative bacteria (Rasool et al., 1992; Zwerdling et al., 2009). Unfortunately, our current understanding of host immune response generated against *B. canis* is mainly based on the studies with smooth *Brucella* species and their mutant rough counterparts. When bacteria penetrate the host, an immediate innate response, mainly mediated by PMNs, is the first line of defense. However, PMNs are not significant in the control of *B. abortus*, even once adaptive immunity has developed, and this strain is resistant to the killing action of PMNs (Barquero-Calvo et al., 2013). In order to assess the serum capacity to enhance phagocytosis and killing of *B. canis*, a versatile assay was designed. As described in previous works with other *Brucella* species, no phagocytosis was observed when bacteria either alone or incubated with control serum was confronted with the neutrophils. When opsonized with vaccinated dog serum, phagocytosis of bacteria was not completely observable by intracellular CFU count, probably because of the rapid destruction immediately after being phagocyted. However, based on the reduction of total bacterial burden, it can be deduced that the free bacteria have been efficiently internalized and killed by the PMN cells. Although individual differences have been observed, the overall bacteriolysis effect ranged from 26 to 52% at 30 minutes of incubation, and 34 to 68% at 75 minutes. Our results indicate that under non-opsonic conditions neutrophils are ineffective in destroying *B. canis*, either confronting cells with bacteria alone or in the presence of negative control serum. Conversely, opsonization with BLSOmp31 vaccinated dog serum not only stimulated binding and uptake of the bacteria but effectively enhanced the destruction of *B. canis*. It has been reported that when

phagocytosis takes place in the absence of heat-labile complement components or specific antibodies, it appears that the absence of the O-chain enhances the uptake of rough LPS phenotypes of *B. abortus*. In fact, the stimulation of oxidative metabolism in bovine neutrophils by *B abortus* is dependent on the presence of bacterial associated opsonins (Canning et al., 1988; Rittig et al., 2003). Based on our results, we could suppose that opsonization would allow phagocytic cells to develop an efficient response that enhances the ability to kill virulent *B. canis*, as observed with smooth strains.

Bactericidal and opsonic antibodies against BLSOmp31 induced in dogs might have a protective role during bacteraemia before the entry of *B. canis* in their target cells. In addition, preputial antibodies could prevent *B. canis* colonization of the reproductive tract.

Although in this study we used a very small number of animals, in future investigations we will use an appropriate number of dogs with the aim to evaluate immune response induced by different formulations and delivery strategies of BLSOmp31 in field conditions.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-Argentina) (to SME). AGD is recipient of a postdoctoral fellowship from CONICET (Argentina). MC, VZ, FAG and SME are members of the Research Career of CONICET. We thank Alberto Bujacich (Royal Canin) who kindly provided dog food, MV Pablo Nejamkin and Dr María José Del Sole (U.N.C.P.B.A, Argentina) for veterinary support and MV Paula Dominguez (CIVETAN-CONICET-CIC) for animal care. Conceived and designed the experiments: SME and MC. Performed the experiments: MC, SME, AGD. Contributed reagents/materials/analysis tools: SME, FAG.

Wrote the paper: MC and SME. FAG, AGD, VZ critically revised the manuscript. All authors read and approved the final manuscript.

References

Barquero-Calvo, E., Martirosyan, A., Ordoñez-Rueda, D., Arce-Gorvel, V., Alfaro-Alarcón, A., Lepidi, H., Malissen, B., Malissen, M., Gorvel, J.-P., Moreno, E., 2013. Neutrophils exert a suppressive effect on Th1 responses to intracellular pathogen *Brucella abortus*. PLoS Pathog 9, e1003167.

Bosseray, N., Plommet, M., 1990. *Brucella suis* S2, *Brucella melitensis* Rev. 1 and *Brucella abortus* S19 living vaccines: residual virulence and immunity induced against three *Brucella* species challenge strains in mice. Vaccine 8, 462-468.

Canning, P., Deyoe, B., Roth, J., 1988. Opsonin-dependent stimulation of bovine neutrophil oxidative metabolism by *Brucella abortus*. American journal of veterinary research 49, 160-163.

Carson, C., Antoniou, M., Ruiz-Argüello, M.B., Alcami, A., Christodoulou, V., Messaritakis, I., Blackwell, J.M., Courtenay, O., 2009. A prime/boost DNA/Modified vaccinia virus Ankara vaccine expressing recombinant *Leishmania* DNA encoding TRYP is safe and immunogenic in outbred dogs, the reservoir of zoonotic visceral leishmaniasis. Vaccine 27, 1080-1086.

Cassataro, J., Pasquevich, K.A., Estein, S.M., Laplagne, D.A., Velikovsky, C.A., de la Barrera, S., Bowden, R., Fossati, C.A., Giambartolomei, G.H., Goldbaum, F.A., 2007. A recombinant subunit vaccine based on the insertion of 27 amino acids from Omp31 to the N-terminus of BLS induced a similar degree of protection against *B. ovis* than Rev. 1 vaccination. Vaccine 25, 4437-4446.

Clausse, M., Díaz, A.G., Ghersi, G., Zylberman, V., Cassataro, J., Giambartolomei, G.H., Goldbaum, F.A., Estein, S.M., 2013. The vaccine candidate BLSOmp31 protects mice against *Brucella canis* infection. Vaccine 31, 6129-6135.

Clausse, M., Díaz, A.G., Ibañez, A.E., Cassataro, J., Giambartolomei, G.H., Estein, S.M., 2014. Evaluation of the Efficacy of Outer Membrane Protein 31 Vaccine Formulations for Protection against Brucella canis in BALB/c Mice. Clinical and Vaccine Immunology 21, 1689-1694.

Clausse, M., Estein, S., 2011. Evaluación de un modelo de infección murino para la evaluación de vacunas homólogas y heterólogas contra *Brucella canis*. InVet 13, 53-60.

Díaz, A.G., Quinteros, D.A., Llabot, J.M., Palma, S.D., Allemandi, D.A., Ghersi, G., Zylberman, V., Goldbaum, F.A., Estein, S.M., 2016. Spray dried microspheres based on chitosan: A promising new carrier for intranasal administration of polymeric antigen BLSOmp31 for prevention of ovine brucellosis. Materials Science and Engineering: C.

Estein, S.M., Fiorentino, M.A., Paolicchi, F.A., Clausse, M., Manazza, J., Cassataro, J., Giambartolomei, G.H., Coria, L.M., Zylberman, V., Fossati, C.A., 2009. The polymeric antigen BLSOmp31 confers protection against *Brucella ovis* infection in rams. Vaccine 27, 6704-6711.

Hampton, M.B., Vissers, M., Winterbourn, C.C., 1994. A single assay for measuring the rates of phagocytosis and bacterial killing by neutrophils. Journal of leukocyte biology 55, 147-152.

HogenEsch, H., 2002. Mechanisms of stimulation of the immune response by aluminum adjuvants. Vaccine 20, S34-S39.

Laplagne, D.A., Zylberman, V., Ainciart, N., Steward, M.W., Sciutto, E., Fossati, C.A., Goldbaum, F.A., 2004. Engineering of a polymeric bacterial protein as a scaffold for the multiple display of peptides. PROTEINS: Structure, Function, and Bioinformatics 57, 820-828.

Lee, H.K., Ha, Y.M., Kim, J.Y., Chae, M.J., Kang, S.I., Her, M., Jung, J.H., 2011. Selection of protective antigen from *Brucella canis* for DNA vaccine Brucellosis International Reserch Conference. Including the 64th Brucellosis Reasearch Conference. Asociación Argentina de Microbiología, Buenos Aires, p. 133.

Lucero, N.E., Corazza, R., Almuzara, M.N., Reynes, E., Escobar, G.I., Boeri, E., Ayala, S.M., 2010. Human *Brucella canis* outbreak linked to infection in dogs. Epidemiology and infection 138, 280.

Margni, R.A., 1996. Apéndice I Inmunología e Inmunoquímica. Fundamentos. Editorial Médica Panamericana, Buenos Aires, Argentina.

Marzetti, S., Carranza, C., Roncallo, M., Escobar, G.I., Lucero, N.E., 2013. Recent trends in human *Brucella canis* infection. Comparative immunology, microbiology and infectious diseases 36, 55-61.

Palomares-Resendiz, E., Arellano-Reynoso, B., Hernández-Castro, R., Tenorio-Gutiérrez, V., Salas-Téllez, E., Suárez-Güemes, F., Díaz-Aparicio, E., 2012. Immunogenic Response of *Brucella canis* virB10 and virB11 Mutants in a Murine Model. Frontiers in Cellular and Infection Microbiology 2.

Pollak, C.N., Wanke, M.M., Estein, S.M., Delpino, M.V., Monachesi, N.E., Comercio, E.A., Fossati, C.A., Baldi, P.C., 2015. Immunization with *Brucella* VirB proteins reduces organ colonization in mice through a Th1-type immune response and elicits a similar immune response in dogs. Clinical and Vaccine Immunology 22, 274-281.

Rasool, O., Freer, E., Moreno, E., Jarstrand, C., 1992. Effect of Brucella abortus lipopolysaccharide on oxidative metabolism and lysozyme release by human neutrophils. Infection and Immunity 60, 1699-1702.

Rittig, M.G., Kaufmann, A., Robins, A., Shaw, B., Sprenger, H., Gemsa, D., Foulongne, V., Rouot, B., Dornand, J., 2003. Smooth and rough lipopolysaccharide phenotypes of *Brucella* induce different intracellular trafficking and cytokine/chemokine release in human monocytes. Journal of leukocyte biology 74, 1045-1055.

Velikovsky, C.A., Goldbaum, F.A., Cassataro, J., Estein, S., Bowden, R.A., Bruno, L., Fossati, C.A., Giambartolomei, G.H., 2003. *Brucella* lumazine synthase elicits a mixed Th1-Th2 immune response and reduces infection in mice challenged with *Brucella abortus* 544 independently of the adjuvant formulation used. Infection and Immunity 71, 5750-5755.

Wanke, M.M., 2004. Canine brucellosis. Animal Reproduction Science 82, 195-207.

Welborn, L.V., DeVries, J.G., Ford, R., Franklin, R.T., Hurley, K.F., McClure, K.D., Paul, M.A., Schultz, R.D., 2011. 2011 AAHA canine vaccination guidelines. Journal of the American Animal Hospital Association 47, 1-42.

Zwerdling, A., Delpino, M.V., Pasquevich, K.A., Barrionuevo, P., Cassataro, J., Samartino, C.G., Giambartolomei, G.H., 2009. *Brucella abortus* activates human neutrophils. Microbes and infection 11, 689-697.

Zylberman, V., Craig, P.O., Klinke, S., Braden, B.C., Cauerhff, A., Goldbaum, F.A., 2004. High order quaternary arrangement confers increased structural stability to *Brucella* sp. lumazine synthase. Journal of Biological Chemistry 279, 8093-8101.

Figure captions

Figure 1: Kinetics of the humoral immune response (IgG) in dogs elicited after immunization with BLSOmp31-AH gel adjuvant. Specific antibodies against BLSOmp31 were evaluated by iELISA in serum **A)** or preputial secretion **B**). The arrows indicate immunization times.

Figure 2: *In vitro* characterization of Th1/Th2 immune response against BLSOmp31 at week 13 after the first immunization. **A)** Specific IgG1 and IgG2 antibodies were evaluated by iELISA. "Control" stands for the unvaccinated dog. The cutoff value for the assay was calculated as the mean O.D. plus 3 standard deviations (S.D.) from 20 sera from THE non-immunized dogs. **B)** Determination of IFN-γ levels (picograms /mL) in supernatants of whole blood cell cultures stimulated with BLSOmp31 (20 μg/mL), with Pokeweed mitogen (10 μg/well) or PBS alone. Each bar represents the geometric means with standard errors of the means of the responses of each dog run in duplicate.

Figure 3: Bactericidal activity of dog sera against *B. canis* with or without a source of complement (canine serum) after 2 h of incubation. % Killing = 100×1− (the mean number of CFU mL^{-1} after incubation/mean number CFU mL^{-1} before incubation). As positive control, an RSAT positive dog serum was used. Asterisks indicate significant difference from control (no serum) * $p<0.05$, *** $p<0.001$.

Figure 4: Changes in extracellular bacteria during the phagocytosis and killing by neutrophils of *B. canis* incubated alone or with sera of vaccinated animals (Dog 1 to 4) or the unvaccinated dog (control dog). The bacteria-neutrophil proportion was 100:1. Samples

were taken at time 0 and after 30 and 75 minutes of incubation, according to the outlined protocol. % extracellular CFU/mL = 100×1 − (the mean number of CFU/mL^{-1} after incubation/mean number CFU/mL^{-1} at time 0). Each line bar represents standard deviation from quadruplicate culture plates of each time sample. * $p < 0.05$ and ** $p < 0.01$ compared with time 0 bacterial count (100%).

Figure 1

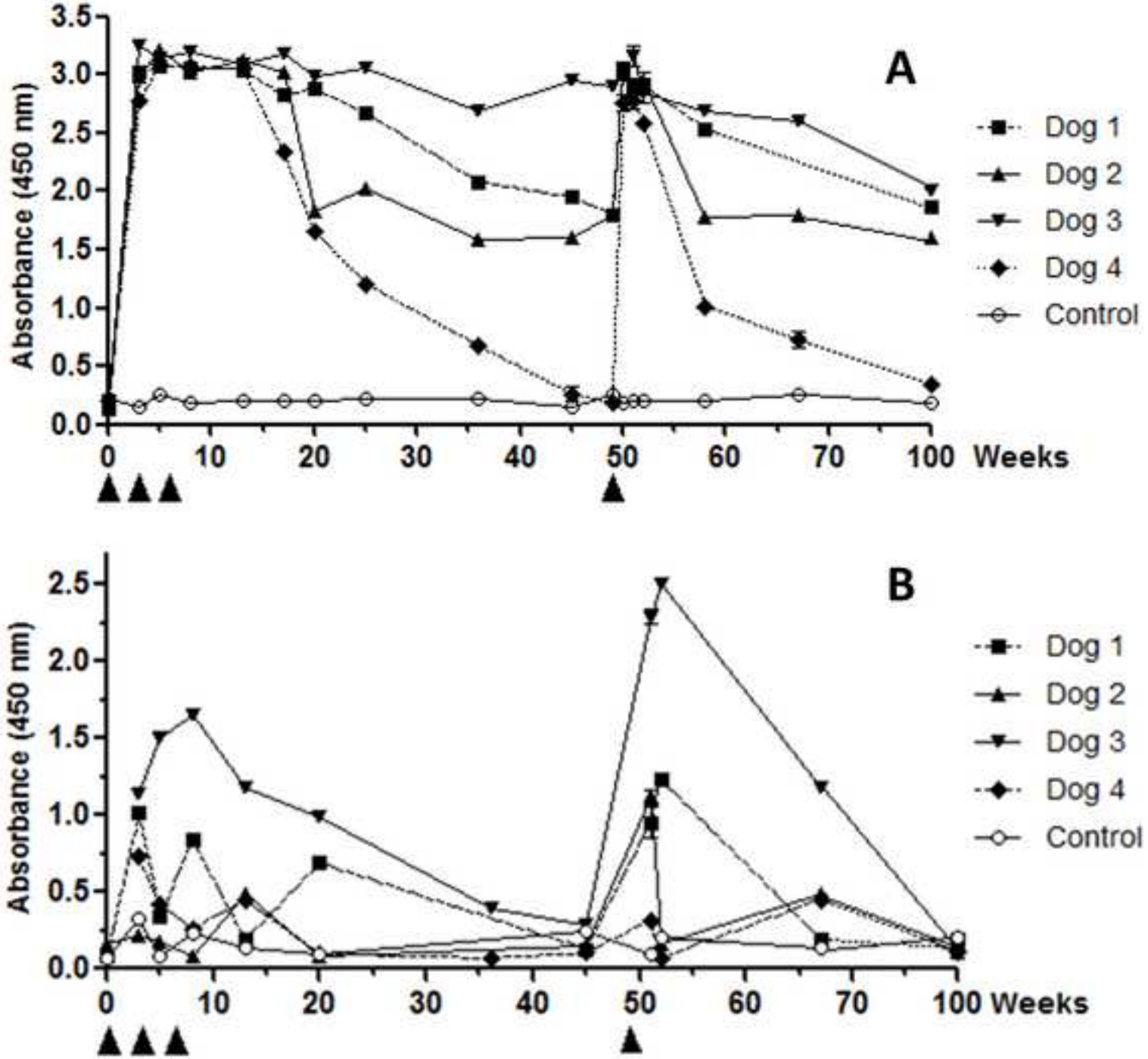

A
Absorbance (450 nm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
100
Weeks
Dog 1
Dog 2
Dog 3
Dog 4
Control
B
Absorbance (450 nm)
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
100
Weeks
Dog 1
Dog 2
Dog 3
Dog 4
Control

Figure 2

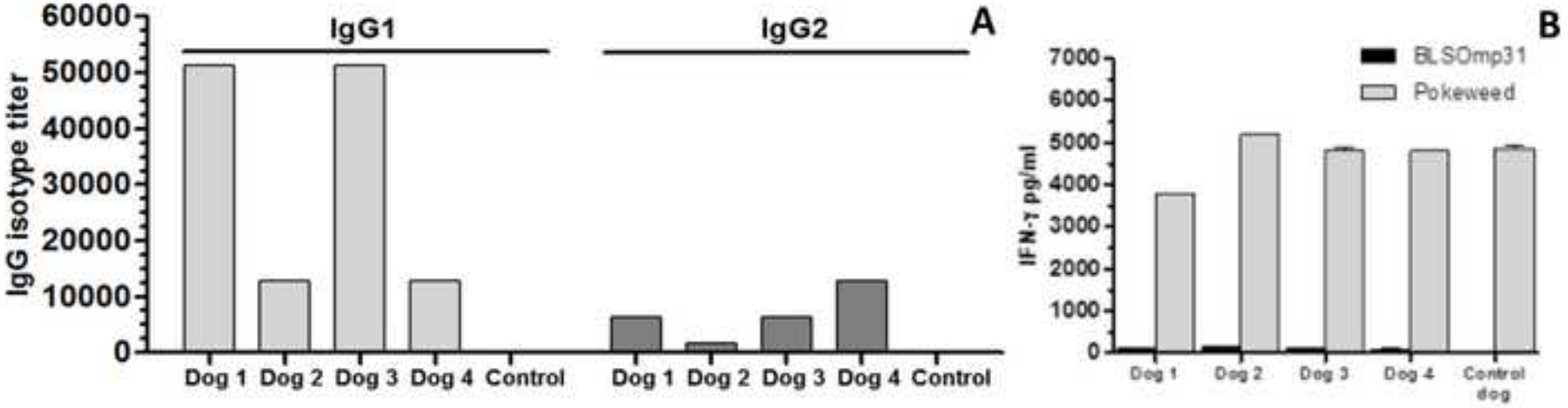

IgG1
IgG2
A
IgG isotype titer
60000
50000
40000
30000
20000
10000
0
Dog 1 Dog 2 Dog 3 Dog 4 Control
Dog 1 Dog 2 Dog 3 Dog 4 Control
B
BLSOmp31
Pokeweed
IFN-γ pg/ml
7000
6000
5000
4000
3000
2000
1000
0
Dog 1
Dog 2
Dog 3
Dog 4
Control dog

Figure 3

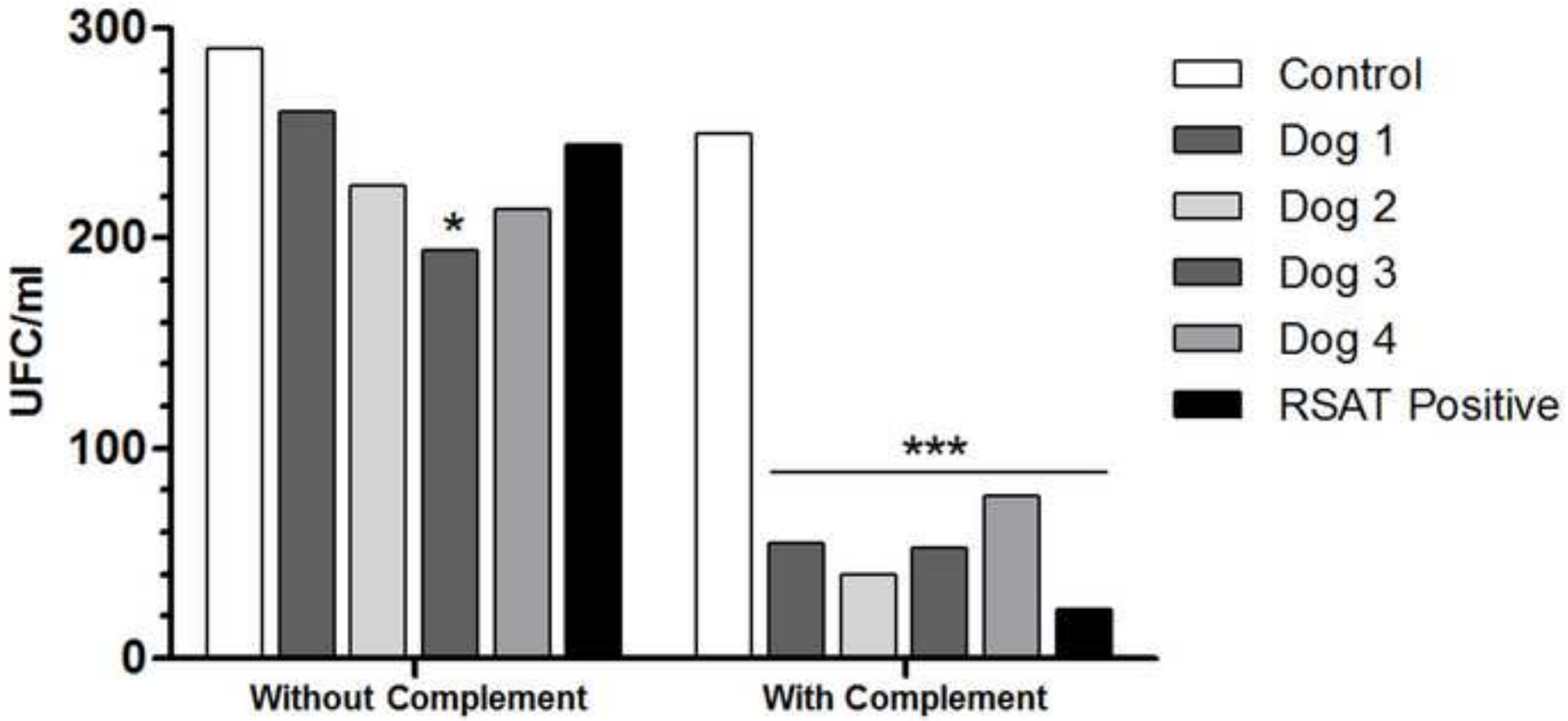
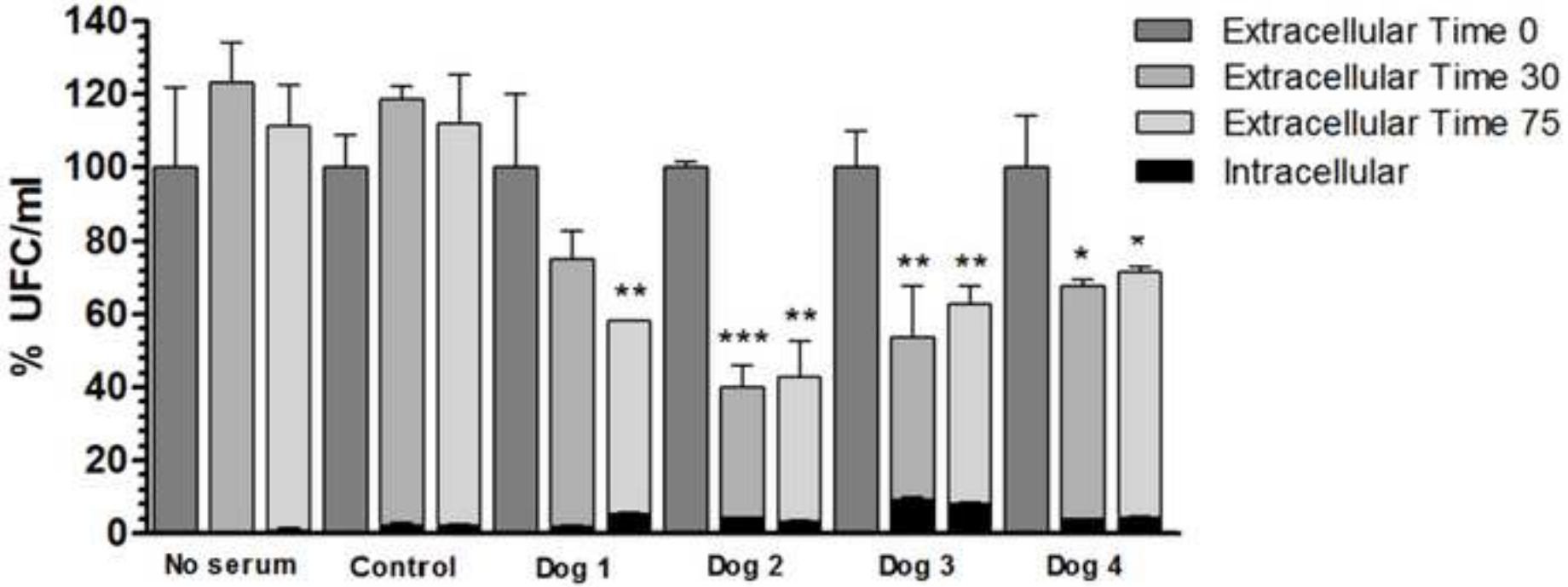

300
200
100
0
UFC/ml
*
***
Without Complement
With Complement
Control
Dog 1
Dog 2
Dog 3
Dog 4
RSAT Positive

Figure 4


% UFC/ml
140
120
100
80
60
40
20
0
No serum
Control
Dog 1
Dog 2
Dog 3
Dog 4
Extracellular Time 0
Extracellular Time 30
Extracellular Time 75
Intracellular
**
***
**
**
**
*
*