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Influence of adjuvant and antigen dose on protection induced by an inactivated whole vaccine against *Neospora caninum* infection in mice.

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ABSTRACT

In this study, the protection afforded by a *Neospora caninum* inactivated vaccine formulated with three different adjuvants (water-in-oil emulsion, aluminum hydroxide with CpG oligodeoxynucleotides and aluminum hydroxide with ginseng extract) and three different parasite doses (10^5, 5×10^5 or 10^6 inactivated whole tachyzoites) was evaluated using a mouse model. Mice were immunized subcutaneously twice at three-week intervals with inactivated Nc-Spain 1H tachyzoites and challenged by intraperitoneal inoculation with 10^6 live Nc-1 tachyzoites. The efficacy of the immunization was evaluated in non-pregnant BALB/c mice on days 1 and 5 (acute infection phase) and days 14 and 30 (chronic infection phase) post-challenge. The results showed the ability of water-in-oil emulsion combined with inactivated 5×10^5 tachyzoites to induce protection against neosporosis during the chronic stage, limiting parasite multiplication in the brain. Aluminum hydroxide-ginseng extract and inactivated tachyzoites reduced the number of parasites circulating in the blood during acute phase but failed to limit the establishment of chronic infection. On the other hand, a dose-effect was observed in groups vaccinated with aluminum hydroxide-ginseng extract in which the lesion severity increased as the inactivated tachyzoite dose. This study demonstrates that efficacy can significantly vary depending on the adjuvant, the dose of antigen and the phase of *N. caninum* infection in which the vaccine is tested.

Key words: *Neospora caninum* / inactivated vaccine/ mice /adjuvant

1. Introduction

Neospora caninum is cyst-forming coccidian parasite that is recognized worldwide as a cause of reproductive failure in cattle (Dubey et al., 2007). Neosporosis is generally latent and asymptomatic in non-pregnant cattle, although the consequences of infection in a pregnant cow can be abortion, birth of a weak calf or birth of a clinically healthy but persistently infected calf (Innes et al., 2002). The development of an effective and safe vaccine against bovine neosporosis is of great importance today due to the significance of the economic losses in the dairy and beef industries (Dubey et al., 2007).

Immunization with live tachyzoites has been reported to confer excellent protection in mice (Miller et al., 2005) and cattle (Williams et al., 2007). However, live parasite vaccines can have problems with safety and short shelf-life; thus the development of an inactivated vaccine could be a practical option for the control of bovine neosporosis. In the last few years, antigen extracts of *N. caninum* tachyzoites and recombinant antigens have been tested in mice, displaying some success at inducing a protective immune response (Innes and Vermeulen, 2006).

The use of potent adjuvants that can boost antigen immunogenicity and induce an appropriate immune response is a critical factor in designing inactivated formulations. In particular, it is one of the main goals in devising vaccines against intracellular protozoan infections such as neosporosis, protection from which cell-mediated immunity (CMI) plays a significant role (Innes et al., 2002). Traditional adjuvants such as emulsions and aluminum hydroxide have been widely tested in vaccine trials against several protozoa such as *Plasmodium falciparum* (Coler et al., 2009), *Toxoplasma gondii* (Petersen et al., 1998; Martin et al., 2004) and *Leishmania major* (Tonui et al., 2004). It has been suggested that the mechanism of action of these adjuvants is

primarily the slow release of antigen into draining lymph (Brewer and Alexander, 1997). Both types of adjuvant have also been proposed to enhance antigen uptake, activate innate immune pathways and induce local recruitment of immune cells, thereby generating an immunocompetent environment at the injection site (Tritto et al., 2009). Administration of water-in-oil (W/O) emulsions stimulates antibody producing plasma cells and also enhances cellular immune responses (Aucouturier et al., 2001; Aguilar and Rodriguez, 2007). On the other hand, aluminum hydroxide has been associated with the induction of high levels of antibodies and Th2-type responses when used alone (Lindblad, 2004). However, when co-administered with other adjuvants such as oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN) or saponins from ginseng, aluminum hydroxide may synergistically enhance the immune response, inducing both Th1- and Th2-type immune responses (Mutwiri et al., 2004; Sun et al., 2008).

Antigen dose has been shown to influence both the type of immune response and the production of cytokines (Hosken et al., 1995), which may influence the efficacy of a vaccine. In neosporosis, no studies focused on antigen dose influence has been carried out, but this factor seems to be important since high parasite doses has been suggested to exacerbate cerebral infections (Baszler et al., 2000).

Because the adjuvant and dose of antigen may influence the development of a protective immune response, the present study was carried out to evaluate different combinations of adjuvants (water-in-oil emulsion and aluminum hydroxide plus CpG-ODN or ginseng extract) and doses of antigen (low, medium, high) for the development of protective efficacy using an inactivated vaccine against *N. caninum* in a BALB/c mouse model.

2. Material and methods

2.1. Mice

Eight-week old female BALB/c mice were obtained from a commercial supplier (Harlan Interfauna Ibérica, Spain). They were free of common viral, parasite and bacterial pathogens according to the results of routine screening procedures performed by the manufacturer. Mice were fed *ad libitum*, in a controlled environment with a 12-hour light and 12-hour dark cycle. All mouse handling procedures complied with EU legislation.

2.2. Parasites and antigen

Two different *N. caninum* isolates were used: Nc-Spain 1H, originally obtained from the brain of a naturally infected asymptomatic calf (Rojo-Montejo et al., 2009b) was used for immunization, and Nc-1 (Dubey et al., 1988) was used for the challenge dose. Both isolates were maintained under the same conditions in a continuous passage of Marc-145 cells as described previously (Perez-Zaballos et al., 2005). Nc-Spain 1H tachyzoites were washed three times in sterile phosphate-buffered saline (PBS, pH 7.4), separated from host cell debris by passing the mixture through a 25-gauge needle, followed by passage through a PD-10 column (Amersham Biosciences, Sweden). The average number of tachyzoites was determined by counting five aliquots using a Neubauer chamber (standard error of the mean 5%). Then, Nc-Spain 1H purified tachyzoites were inactivated with 0.01 M (final concentration) binary ethylenimine (BEI) for a period of 96 h at 4°C, followed by neutralization with sodium thiosulfate (Andrianarivo et al., 2000).

For challenge, Nc-1 tachyzoites were harvested, and viability was determined by Trypan blue exclusion followed by counting in a Neubauer chamber. The organisms

were adjusted to a concentration of 10^6 tachyzoites in a final volume of 200 µl/mouse and used immediately to infect the mice.

Nc-1 tachyzoites for PCR controls were purified as described above, pelleted by centrifugation (600 × *g*, 10 min) and frozen at -80ºC until use. To obtain soluble *N. caninum* protein antigen, purified Nc-1 tachyzoites were suspended in 1 ml of 10 mM Tris-HCl containing 2 mM phenylmethylsulfonyl fluoride (Sigma, USA) and disrupted by ultrasound treatment (Sonifier 450, Branson Ultrasonic, USA) in an ice-bath. Cell debris and unlysed cells were removed by centrifugation (10,000 × *g*, 20 min, 4ºC). Supernatant protein was quantified using the Micro BCA protein assay (Pierce, USA), and then the supernatant was aliquoted and frozen at -80ºC until use.

2.3. Adjuvants

Inactivated whole Nc-Spain1H tachyzoites were incorporated into a water-in-oil emulsion (adjuvant A), aluminum hydroxide with CpG oligodeoxynucleotides (adjuvant B) or aluminum hydroxide with ginseng extract (adjuvant C). In the adjuvant A, the emulsion was used at a concentration of 104 mg per dose. In adjuvants B and C, aluminum hydroxide was used at a concentration of 1.53 mg per dose, combined with 0.01 mg of CpG oligodeoxynucleotides or 0.4 mg of ginseng extract, respectively. All the adjuvant preparations were developed by HIPRA (Girona, Spain). The immunizing doses were prepared in a final volume of 200 µl per mouse.

2.4. Experimental design and sampling

BALB/c mice were divided into 17 groups, each consisting of 20 animals. Each of the mice was subcutaneously injected with either 10^5, 5×10^5 or 10^6 inactivated whole Nc-Spain1H tachyzoites incorporated into adjuvant A, B, C or phosphate-buffered saline (PBS). Other groups received adjuvant A, B, C or PBS alone (Table 1). The mice were immunized subcutaneously twice at three-week intervals and sublethally challenged

intraperitoneally (i.p.) three weeks after the last immunization with 10^6 live Nc-1 tachyzoites. The animals were monitored daily for the presence of clinical signs of neosporosis. Evaluation of clinical signs was based on previous *N. caninum* infection studies (Atkinson et al., 1999; Eperon et al., 1999; Collantes-Fernandez et al., 2006) . Five randomly selected animals from each group were sacrificed on days 1 and 5 (acute infection stage) and days 14 and 30 (chronic infection stage) post-challenge. Blood samples were collected by cardiac puncture in EDTA tubes, centrifuged and plasma was recovered and cryopreserved at -80ºC for antibody analysis. Pelleted EDTA-blood cells were stored at 4ºC until DNA extraction. Brain and lung samples were aseptically recovered and frozen at -80ºC until they were analyzed by PCR. On days 14 and 30 post-challenge, one brain hemisphere was fixed in 10% neutral buffered formalin solution for processing by routine histological methods.

2.5. DNA extraction and ITS1 nested-PCR

A Real Pure Extraction genomic DNA kit (Durviz, Spain) was employed to extract DNA from tachyzoites and 10-20 mg of host tissues, and the Real Pure DNA Extraction SSS (Durviz, Spain) to extract DNA from blood samples according to the manufacturers' protocols. The quantity of DNA was measured spectrophotometrically, and samples were diluted to a final concentration of 40 ng/µl for DNA detection by nested-PCR and quantification by real time-PCR. For detection of parasite DNA, a nested-PCR on the internal transcribed spacer (ITS1) region of *N. caninum* was carried out with four oligonucleotides as described by Buxton et al. (Buxton et al., 1998). Secondary amplification products were visualized by 1.8% agarose gel electrophoresis and ethidium bromide staining. To avoid false positive reactions, DNA extraction, PCR sample preparation and electrophoresis were performed in separate rooms employing different sets of instruments, aerosol barrier tips and disposable gloves. Moreover,

negative control samples were included in each set of DNA extractions and PCR reactions.

2.6. Evaluation of parasite burden by real-time PCR

N. caninum DNA in positive nested-PCR samples was quantified by real-time PCR as described previously (Collantes-Fernandez et al., 2002) using an ABI 7300 Prism Sequence Detector Machine (Applied Biosystems, USA) and the commercial kit Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, U.K.). Oligonucleotide primers pairs from the *N. caninum* Nc5 sequence that amplify a 76-bp DNA fragment were used to quantify parasite load, and for the quantification of host DNA, specific fragment of 71 bp was amplified from the 28S rRNA gene (Collantes-Fernandez et al., 2002). Samples were run in duplicate in separate tubes. *N. caninum* organisms were quantified by interpolation of Ct values (cycle threshold: the fractional cycle number reflecting a positive PCR result) on a standard curve from DNA equivalent to 10^{-1}- 10^{4} tachyzoites. The amount of DNA per sample was normalized by quantification of the 28S rRNA gene, and a standard curve was generated with five-fold serial dilutions of murine DNA quantified by UV spectrophotometry. The data were analyzed with Sequence Detection System Software v.1.6 (Applied Biosystems, USA). Parasite load was expressed as parasite number/µg host DNA.

2.7. Histopathological analysis

Multiple sections of different regions of the brain were examined by routine histological methods. Tissues fixed in 10% neutral formalin and dehydrated through graded alcohols were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Analysis was based on the observation of lesions characteristic of or consistent with *N. caninum* infection in the brain (Lindsay et al., 1995; Collantes-Fernandez et al., 2004). Lesions in the brain were assessed according to the severity of inflammation and the extension of

affected tissue in each section, and lesion scores were assigned on a three-point scale according to the following scheme: no lesion (= 0); mild meningitis, perivascular cuffing and gliosis (= 1); moderate meningitis, perivascular cuffing, gliosis, mild glial nodules and focal granulomas (= 2); or severe lesions including meningitis, perivascular cuffing, gliosis, glial nodules and multifocal granulomas (= 3) (Pereira Garcia-Melo et al., 2010). The mean of these values was determined for each animal and a median lesion score corresponding to each group was represented.

2.8. Humoral immune responses

N. caninum-specific serum isotypes IgG2a and IgG1 were determined by ELISA. Briefly, 96-well plates were coated with soluble *N. caninum* tachyzoite antigen (0.5 µg in 100 µl/well), and diluted murine serum samples (1:100) and anti-mouse IgG2a or IgG1 antibody (1:5,000; Southern Biotechnology, USA) were used as described previously (Collantes-Fernandez et al., 2006).

2.9. Statistical analysis

No significant differences were found in frequency of parasite detection between the two time points per infection phase. Consequently, to make the statistical analysis more consistent by increasing the number of animals per group, data collected within a phase were pooled (on days 1 and 5 p.i. for acute phase and on days 14 and 30 for chronic phase). All immunized groups were compared to the non-immunized/challenged group to evaluate the protective efficacy of the vaccine. The influence of the adjuvant type and killed *N. caninum* tachyzoites dose was evaluated by comparing the different adjuvants at the same antigen dose and the different antigen doses with the same adjuvant group, respectively.

Frequencies of parasite detection were compared by Fisher´s exact test. The parasite load and lesion scores were analyzed using the Kruskal-Wallis test followed by a non-

parametric multiple-comparison test. When a statistically significant difference was obtained using the Kruskal-Wallis test but the multiple comparison test failed to reveal it, the results obtained by the Kruskal-Wallis test were preferred as indicated by Morrison et al. (2002). Serological data were compared using one-way ANOVA followed by Duncan´s Multiple Range test. All statistical analyses were performed using STATGRAPHICS Plus 4.1 (StatPoint, USA).

3. Results

3.1. Clinical signs

Clinical signs compatible with *N. caninum* infection (inactivity, rough coat and pelvic limb weakness) were observed in the fourth week post-challenge (chronic stage) in very few animals. Specifically, one mouse each from groups 10 (C/5×10^5) and 12 (C/PBS) displayed clinical signs.

3.2. Parasite detection in the blood, lungs and brain

The presence of *N. caninum* DNA in blood and lung was more often detected during the acute infection phase (days 1-5 post-challenge), whereas in brain was mainly observed in the chronic infection phase (days 14-30 post-challenge), (Table 2).

Significant differences during acute phase were only observed in parasitaemia. In particular, the immunization with C/5×10^5 (group 10) reduced significantly the parasitaemia compared to non-immunized/challenged animals (group 16) (P<0.05). When the adjuvants were compared, the number of animals with parasitaemia was lower in group vaccinated with C/5×10^5 (group 10) than in those inoculated with A/5×10^5 (group 2), B/5×10^5 (group 6) and PBS/5×10^5 (group 14) (P<0.05). The effect of antigen dose was observed in groups given adjuvant C. Particularly, parasite presence in

blood was lower in animals immunized with C/5×10^5 (group 10) than in those inoculated with C/PBS (group 12) (P<0.05).

During chronic stage (days 14-30 post-challenge), parasitaemia was transient in all groups and no significant differences were found. The parasite presence in the lungs was low in most groups. However, when the influence of adjuvant was evaluated, significant increased parasite presence was detected in lungs from the group given C/10^6 (group 11) compared with group vaccinated with A/10^6 (group 3) (P<0.01). In addition, parasite DNA was more often detected in groups immunized with inactivated tachyzoites plus PBS than in animals vaccinated with adjuvant A either with 5×10^5 or 10^6 inactivated tachyzoites (groups 14, 15 versus 2, 3; P<0.05). In the brain, no positive animals were found in group 2 (A/5×10^5) in which the parasite presence was significantly lower than in non-immunized/challenged animals (group 16) (P<0.01). An adjuvant-dependent effect was detected in groups immunized with adjuvant A or B. Particularly, parasite DNA was demonstrated in a lower number of animals given A/10^5 and A/5×10^5 than in groups immunized with C/10^5, C/5×10^5, PBS/5×10^5 (groups 1 *vs.* 9; 2 *vs.* 10, 14; P<0.05). Immunization of mice with adjuvant B combined with 10^6 inactivated tachyzoites also significantly reduced the parasite presence compared with group vaccinated with C/10^6 (group 11) (P<0.05). The effect of antigen dose was observed in groups given A/5×10^5 and B/10^6 in which parasite presence was significantly reduced compared with groups vaccinated with the adjuvant-control groups (A/PBS and B/PBS) (P<0.05).

These results indicate that immunization with C/5×10^5 lead to the reduction of parasitaemia in the acute infection phase whereas immunization with A/5×10^5 efficiently limited the establishment of infection in brain.

3.3. Evaluation of parasite burden

On days 1-5 post-challenge, significant differences were observed in blood and groups vaccinated with adjuvant C which showed a lower parasitaemia level (P<0.05) (Figure 1A). The immunization with C/5×10^5 (group 10) reduced significantly the parasitaemia compared to non-immunized/challenged animals (group 16) (P<0.05). When we determined the adjuvant effect, animals vaccinated with C/5×10^5 (group 10) reduced parasitaemia level compared with B/5×10^5 (group 6) and PBS/5×10^5 (group 14) (P<0.01) (Figure 1).

On days 14-30 post-challenge, animals immunized with adjuvant C and 10^6 inactivated tachyzoites had greater parasite loads in the lungs (group 11 vs. group 3; P<0.05) (Figure 1B). In the brain, mice vaccinated with C/10^6 had an even higher parasitic load than the non-immunized/challenged animals (P<0.05). The effect of adjuvant on the reduction of brain parasite loads was observed in groups vaccinated with adjuvant A or B (P<0.01) (Figure 1C). Specifically, groups vaccinated with A/5×10^5 or B/10^6 showed significantly lower parasite burdens than those immunized with adjuvant C/5×10^5, C/10^6 and PBS/5×10^5 (2 vs. 10, 14; 7 vs. 11; P<0.01). A dose-dependent effect was observed in groups immunized with adjuvant C in which an increase in the parasite load was associated with a higher dose of inactivated tachyzoites (11 vs. 12, P<0.01).

Altogether, these data show the ability of adjuvant C and inactivated tachyzoites to reduce the number of parasites circulating in the blood during acute phase but not to limit the establishment of chronic infection in brain. On the other hand, immunization with adjuvant A or B and inactivated tachyzoites displayed the lowest brain parasite burden in the chronic infection phase.

3.4. Histopathology

In groups vaccinated with inactivated tachyzoites and adjuvant C and PBS, histopathological examination of brain tissue from mice sacrificed on days 14-30 post-challenge revealed the presence of large severe necrotic foci with mononuclear infiltrates. A few animals, specifically from the PBS/5×10^5 and C/10^6 groups, showed foci of dystrophic calcification in the necrotic areas. Animals vaccinated with adjuvants A or B and non-immunized/challenged animals had mild to moderate microgliosis, perivascular cuffs or no pathology. The statistical analysis showed that mice vaccinated with adjuvant C or PBS combined with higher tachyzoite doses had higher lesion severity scores than the non-immunized/challenged animals (group 11 and group 14 versus group 16; $P<0.01$). Regarding adjuvant influence, immunization with adjuvants A or B combined with higher inactivated parasite doses reduced the severity of lesions compared with animals immunized with PBS (groups 2, 6 versus 14) or adjuvant C (groups 3, 7 versus 11) ($P<0.01$) (Figure 2). In addition, a dose-effect was observed in groups vaccinated with adjuvant C in which the lesion severity increased as the inactivated tachyzoite dose increased (group 9, 12 versus 11; $P<0.01$).

Taken together these results show that adjuvant C or PBS plus high doses of inactivated tachyzoites not only do not confer protection but also exacerbate the cerebral neosporosis.

3.5. Humoral immune response

Production of IgG1 was predominant during the experiment in the vaccinated groups.

On days 1-5 post-challenge (Figure 3A)**,** adjuvant A with 5×10^5 or 10^6 inactivated tachyzoites induced higher levels of IgG1 and IgG2a isotypes than mice vaccinated with adjuvants B, C or PBS plus inactivated tachyzoites and than non-immunized/challenged animals ($P<0.05$). An increase in antibody concentration was also observed in animals vaccinated with PBS and inactivated tachyzoites (groups 14 and 15) compared with

adjuvants B and C (P<0.0001). In addition, a parasite dose effect was observed since the higher inactivated tachyzoites doses stimulated higher IgG1 and IgG2a values (P<0.0001).

On days 14-30 post-challenge (Figure 3B), mice vaccinated with inactivated tachyzoites with adjuvant A, B, C and PBS developed higher IgG1 levels than non-immunized/challenged animals during chronic infection (P<0.05). Additionally, mice given adjuvants A, B and C displayed higher IgG1 values than groups inoculated with PBS as well as either 5×10^5 or 10^6 inactivated tachyzoites and control adjuvant groups (P<0.01). However, IgG2a levels were only significantly higher in groups vaccinated with A/10^6 compared with A/10^5 or the adjuvant control group (A/PBS) (P<0.05).

In summary, all challenged groups had higher concentration of IgG1 than IgG2a. Furthermore, immunization with adjuvant A induced the highest antibody levels.

4. Discussion

In designing an inactivated vaccine formulation, the choice of appropriate adjuvants and antigen doses is crucial for a successful vaccination program, and is essentially empiric. Therefore, trial-and-error studies are a useful approach to select vaccine candidates with acceptable safety and proven efficacy. Here, we performed a concurrent study of several adjuvants formulated with the same antigen at different doses, evidencing the role of these factors on protection against *N. caninum* infection.

In addition, the use of a laboratory model for testing vaccine formulations is necessary for the selection of effective protocols prior to their use in cattle. We used a BALB/c mouse model previously developed in our laboratory, in which experimental infections with Nc-1 were characterized by an early phase, during which parasitaemia and parasite DNA was detected mainly in lungs, and a chronic stage with parasite presence in the brain (Collantes-Fernandez et al., 2006). Nc-1 isolate has been widely used as the

challenge isolate in numerous vaccine studies (Lindsay et al., 1999; Lunden et al., 2002; Cannas et al., 2003; Debache et al., 2008; Ribeiro et al., 2009). Inoculation of high doses of Nc-1 tachyzoites in mice has been observed to be lethal, inducing high morbidity and mortality and severe lesions consistent with acute neosporosis (Alaeddine et al., 2005; Ramamoorthy et al., 2006). In order to establish a latent stage of the infection characterized by low mortality, the presence of the parasite in the brain and encephalitis as the main lesion (Dubey and Lindsay, 1996; Collantes-Fernandez et al., 2004), a sublethal challenge dose was utilised. On the other hand, in an attempt to avoid the use of isolates maintained *in vitro* for several years, we used the new isolate Nc-Spain 1H (Rojo-Montejo et al., 2009a; Rojo-Montejo et al., 2009b) with low passage number, because culturing could select for variant parasite populations that do not include antigens or present them incorrectly (Miller et al., 2005). Inactivated whole Nc-Spain 1H tachyzoites were employed to deliver to the immune system with either *N. caninum* organelles or membrane antigens accessible during intracellular or extracellular phases of the infection. Furthermore, inactivated tachyzoites might be a good candidate to use as antigen in vaccine trials since intact inactivated tachyzoites have been shown to induce a greater increase in the number of IFN-γ producing NK cells compared with sonicated soluble antigens (Klevar et al., 2007). Nc-Spain 1H tachyzoites were inactivated with BEI, a chemical method shown to cause fewer epitope changes to the antigen than other treatments (Blackburn and Besselaar, 1991) and that have previously been used in a *N. caninum* inactivated vaccine formulation (Andrianarivo et al., 2000).

Concerning adjuvants, various strategies were chosen. The first approach was to test a standard water-in-oil emulsion (adjuvant A) widely used in ruminants, which primarily acts as a depot. On the other hand, we employed the co-administration of aluminum

hydroxide with novelty adjuvants such as CpG-ODN (adjuvant B) or ginseng extract (adjuvant C), which are able to modify the cytokine network. CpG-ODN is a potent activator of the innate immune response that subsequently amplifies the antigen-specific immune response. The saponins from ginseng extract have been reported to have adjuvant properties and appear to exert a number of effects on the immune system, including enhancing lymphocyte proliferation and stimulating the secretion of a broad range of cytokines (Rivera et al., 2005; Sun et al., 2007; Song and Hu, 2009). The combination of aluminum hydroxide with immunostimulatory substances may synergistically enhance the immune response boosting both cellular (Th1) as well as humoral (Th2) immune responses (Davis et al., 1998; Rivera et al., 2003; Sun et al., 2008).

The results of this study demonstrated that the protective efficacy varied depending on the adjuvant and the stage of *N. caninum* infection in which the vaccine was tested. Thus, water-in-oil emulsion (adjuvant A) combined with whole inactivated tachyzoites induced protection against neosporosis during the chronic stage. Specifically, in mice vaccinated with adjuvant A and 5×10^5 inactivated tachyzoites, no detectable parasite DNA and a reduction in the severity of lesions in the brain were found. It is unclear what immunological mechanisms have been developed by this adjuvant in the protection against cerebral neosporosis. The depot effect proposed as a mechanism of action of W/O emulsions probably induced the significant increase of antibody production observed in mice immunized with adjuvant A. W/O-mediated protection was associated with predominant IgG1 but also high IgG2a levels, suggesting that a Th1/Th2 balance could confer protection against cerebral disease. Debache et al. (2008) also observed reduced cerebral infection in mice after immunization with other W/O emulsions such as Freund´s incomplete adjuvant mixed with recNcROP protein but in

contrast to the present results, the protection was associated with predominant IgG2a levels.

Regarding adjuvant B, the properties of CpG plus aluminum hydroxide combination have been previously demonstrated in vaccine trials against other protozoa (Near et al., 2002; Su et al., 2003). However, no studies have yet been conducted to evaluate the potential of this combination in neosporosis and only the immunostimulatory effect of CpG-ODN has been recently tested against *Neospora*-infection (Ribeiro et al., 2009). In this previous study, the protection induced by CpG-adjuvanted preparations against cerebral infection was primarily influenced by the antigen choice (lysate antigen and excreted-secreted antigens). In the present study, immunization with adjuvant B and 10^6 inactivated tachyzoites reduced parasite presence and burden in brain but significant protection was not observed against non-immunized/challenged group. The partial control of parasite infection induced by adjuvant B constitutes a promising result and is motivation for future studies.

On the other hand, immunization of BALB/c mice with whole inactivated tachyzoites and aluminum hydroxide-ginseng extract (adjuvant C) significantly reduced acute parasitaemia but failed to protect during the chronic stage. Since the degree of parasitaemia is likely to be an important factor in the outcome of *N. caninum* infection in pregnant cattle (Innes et al., 2002; Staska et al., 2003), vaccination with the adjuvant C could control the vertical transmission of the parasite during pregnancy, as it would reduce the parasitaemia and a lower level of parasites would invade the placenta and foetus. However, mice immunized with adjuvant C showed higher brain parasite burden and lesion severity than non-immunized/challenged animals. In contrast, mice immunized with adjuvant C alone had similar parasite levels to non-immunized/challenged animals, showing that the adverse results seen in mice

vaccinated with adjuvant C combined with inactivated tachyzoites could be mainly due to the parasite antigen inoculated. Conversely, our results disagree with a previous study in which the level of parasitaemia appeared to correlate well with disease severity and cerebral parasite loads (Pinitkiatisakul et al., 2008). Immunization with NcSRS2-iscoms decreased the level of parasitaemia, which probably reduced the number of parasites reaching the brain, and this could be the reason for the reduction in brain parasite load and clinical symptoms. The reason why adjuvant C combined with parasite antigen reduced the levels of parasitaemia but which was unsuccessful in protecting during the chronic stage of *N. caninum* infection is unknown. Probably, the immune response induced against rapidly replicating tachyzoites during acute infection could have precipitated parasite survival strategies to evade the immune response. Ginseng extracts have a broad range of immunological activities, including the improvement of phagocytic activity of immune cells (Song and Hu, 2009). Co-administration of ginseng extract and aluminum hydroxide with inactivated tachyzoites may have facilitated the internalization of parasites by migratory cells of the immune system without their neutralization, favouring their rapid dissemination to multiple organs including immunoprivileged tissues such as the brain. Leukocyte trafficking to disseminate intracellular parasites via a Trojan horse-type mechanism has been postulated for other apicomplexan parasites such as *T. gondii* (Lambert et al., 2006).

Unexpectedly, immunization with parasite antigen plus adjuvant B or C induced a low antibody response with a dominance of the IgG1 subclass. The precise immune modulation induced by the adjuvants employed here could not be determined by means of the analysis of *N. caninum*-specific IgG1 and IgG2a production due to a lack of correlation between the protection level and the humoral immune response detected.

Therefore, more research is needed into the mechanisms by which protective immunity can be induced by these formulations.

Understanding how antigen dose influences vaccine efficacy is also important for designing vaccines. To date, several immunization schedules have been tested in *N. caninum* inactivated vaccine trials (Baszler et al., 2000; Lunden et al., 2002; Cannas et al., 2003; Ribeiro et al., 2009) although dose-related effects in inactivated formulations have not been assessed. In the present study, three antigen doses (10^5, 5×10^5 and 10^6 inactivated tachyzoites) were tested. The serological response demonstrated a dose-related immunogenicity in all of the immunized groups. However, with respect to protection, we only observed dose-dependent effects in animals immunized with adjuvant C in the chronic stage, in which parasite loads and lesion severity increased in the target organs as did the antigen dose. Similar findings were found after the use of 50 µg of soluble *N. caninum* tachyzoite antigen that resulted in an exacerbation of the infection in mice (Baszler et al., 2000), whereas immunization of mice with low doses of crude lysate such as the equivalent of 10^5 tachyzoites (approximately 5 µg) or 15 µg reduced congenital transmission and protected completely against cerebral infection, respectively (Liddell et al., 1999; Cannas et al., 2003). Moreover, immunization with inactivated tachyzoites and PBS also enhanced susceptibility in the chronic phase, even though we did not find a significant dose-related effect. Previously, it was reported that mice immunized with parasite lysate alone or excreted-secreted antigens were unprotected and appeared even more susceptible than the control mice, independently of the inoculated parasite dose (Lindsay et al., 1999; Lunden et al., 2002; Ribeiro et al., 2009).

In summary, data from this study demonstrated that the choice of adjuvant is crucial to the efficacy of the inactivated vaccine, but the efficacy can also vary depending on

antigen dose and the stage of *N. caninum* infection in which the vaccine is tested. Aluminum hydroxide-ginseng extract with 5×10^5 inactivated tachyzoites significantly reduced acute parasitaemia, whereas the highest protection efficacy during the chronic stage was observed with the oil-water emulsion with 5×10^5 inactivated tachyzoites, which limited parasite multiplication in brain. This study primarily focused on the efficacy of different vaccine formulations in a laboratory model. We consider this trial-and-error approach a valuable tool for the preliminary selection of promising vaccine protocols against *N. caninum* infection. Further studies are necessary to test the efficacy against congenital neosporosis and for comprehensively evaluating the precise immune modulation induced by these formulations.

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References

Aguilar, J.C., Rodriguez, E.G., 2007. Vaccine adjuvants revisited. Vaccine 25 (19), 3752-3762.

Alaeddine, F., Keller, N., Leepin, A., Hemphill, A., 2005. Reduced infection and protection from clinical signs of cerebral neosporosis in C57BL/6 mice vaccinated with recombinant microneme antigen NcMIC1. J. Parasitol. 91 (3), 657-665.

Andrianarivo, A.G., Rowe, J.D., Barr, B.C., Anderson, M.L., Packham, A.E., Sverlow, K.W., Choromanski, L., Loui, C., Grace, A., Conrad, P.A., 2000. A POLYGEN-adjuvanted killed *Neospora caninum* tachyzoite preparation failed to prevent foetal

infection in pregnant cattle following i.v./i.m. experimental tachyzoite challenge. Int. J. Parasitol. 30 (9), 985-990.

Atkinson, R., Harper, P.A., Ryce, C., Morrison, D.A., Ellis, J.T., 1999. Comparison of the biological characteristics of two isolates of *Neospora caninum*. Parasitology 118 (Pt 4) (Pt 4), 363-370.

Aucouturier, J., Dupuis, L., Ganne, V., 2001. Adjuvants designed for veterinary and human vaccines. Vaccine 19 (17-19), 2666-2672.

Baszler, T.V., McElwain, T.F., Mathison, B.A., 2000. Immunization of BALB/c mice with killed *Neospora caninum* tachyzoite antigen induces a type 2 immune response and exacerbates encephalitis and neurological disease. Clin. Diagn. Lab. Immunol. 7 (6), 893-898.

Blackburn, N.K., Besselaar, T.G., 1991. A study of the effect of chemical inactivants on the epitopes of Rift Valley fever virus glycoproteins using monoclonal antibodies. J. Virol. Methods 33 (3), 367-374.

Brewer, J.M., Alexander, J., 1997. Cytokines and the mechanisms of action of vaccine adjuvants. Cytokines Cell. Mol. Ther. 3 (4), 233-246.

Buxton, D., Maley, S.W., Wright, S., Thomson, K.M., Rae, A.G., Innes, E.A., 1998. The pathogenesis of experimental neosporosis in pregnant sheep. J. Comp. Pathol. 118 (4), 267-279.

Cannas, A., Naguleswaran, A., Muller, N., Eperon, S., Gottstein, B., Hemphill, A., 2003. Vaccination of mice against experimental *Neospora caninum* infection using NcSAG1- and NcSRS2-based recombinant antigens and DNA vaccines. Parasitology 126 (Pt 4), 303-312.

Coler, R.N., Carter, D., Friede, M., Reed, S.G., 2009. Adjuvants for malaria vaccines. Parasite Immunol. 31 (9), 520-528.

Collantes-Fernandez, E., Alvarez-Garcia, G., Perez-Perez, V., Pereira-Bueno, J., Ortega-Mora, L.M., 2004. Characterization of pathology and parasite load in outbred and inbred mouse models of chronic *Neospora caninum* infection. J. Parasitol. 90 (3), 579-583.

Collantes-Fernandez, E., Lopez-Perez, I., Alvarez-Garcia, G., Ortega-Mora, L.M., 2006. Temporal distribution and parasite load kinetics in blood and tissues during *Neospora caninum* infection in mice. Infect. Immun. 74 (4), 2491-2494.

Collantes-Fernandez, E., Zaballos, A., Alvarez-Garcia, G., Ortega-Mora, L.M., 2002. Quantitative detection of *Neospora caninum* in bovine aborted fetuses and experimentally infected mice by real-time PCR. J. Clin. Microbiol. 40 (4), 1194-1198.

Davis, H.L., Weeratna, R., Waldschmidt, T.J., Tygrett, L., Schorr, J., Krieg, A.M., 1998. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J. Immunol. 160 (2), 870-876.

Debache, K., Guionaud, C., Alaeddine, F., Mevissen, M., Hemphill, A., 2008. Vaccination of mice with recombinant NcROP2 antigen reduces mortality and cerebral infection in mice infected with *Neospora caninum* tachyzoites. Int. J. Parasitol. 38 (12), 1455-1463.

Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc. 193 (10), 1259-1263.

Dubey, J.P., Lindsay, D.S., 1996. A review of *Neospora caninum* and neosporosis. Vet. Parasitol. 67 (1-2), 1-59.

Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemiology and control of neosporosis and *Neospora caninum*. Clin. Microbiol. Rev. 20 (2), 323-367.

Eperon, S., Bronnimann, K., Hemphill, A., Gottstein, B., 1999. Susceptibility of B-cell deficient C57BL/6 (microMT) mice to *Neospora caninum* infection. Parasite Immunol. 21 (5), 225-236.

Hosken, N.A., Shibuya, K., Heath, A.W., Murphy, K.M., O'Garra, A., 1995. The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. J. Exp. Med. 182 (5), 1579-1584.

Innes, E.A., Andrianarivo, A.G., Bjorkman, C., Williams, D.J., Conrad, P.A., 2002. Immune responses to *Neospora caninum* and prospects for vaccination. Trends Parasitol. 18 (11), 497-504.

Innes, E.A., Vermeulen, A.N., 2006. Vaccination as a control strategy against the coccidial parasites *Eimeria*, *Toxoplasma* and *Neospora*. Parasitology 133 Suppl, S145-68.

Klevar, S., Kulberg, S., Boysen, P., Storset, A.K., Moldal, T., Bjorkman, C., Olsen, I., 2007. Natural killer cells act as early responders in an experimental infection with *Neospora caninum* in calves. Int. J. Parasitol. 37 (3-4), 329-339.

Lambert, H., Hitziger, N., Dellacasa, I., Svensson, M., Barragan, A., 2006. Induction of dendritic cell migration upon *Toxoplasma gondii* infection potentiates parasite dissemination. Cell. Microbiol. 8 (10), 1611-1623.

Liddell, S., Jenkins, M.C., Collica, C.M., Dubey, J.P., 1999. Prevention of vertical transfer of *Neospora caninum* in BALB/c mice by vaccination. J. Parasitol. 85 (6), 1072-1075.

Lindblad, E.B., 2004. Aluminium adjuvants--in retrospect and prospect. Vaccine 22 (27-28), 3658-3668.

Lindsay, D.S., Lenz, S.D., Blagburn, B.L., Brake, D.A., 1999. Characterization of temperature-sensitive strains of *Neospora caninum* in mice. J. Parasitol. 85 (1), 64-67.

Lindsay, D.S., Lenz, S.D., Cole, R.A., Dubey, J.P., Blagburn, B.L., 1995. Mouse model for central nervous system *Neospora caninum* infections. J. Parasitol. 81 (2), 313-315.

Lunden, A., Wright, S., Allen, J.E., Buxton, D., 2002. Immunisation of mice against neosporosis. Int. J. Parasitol. 32 (7), 867-876.

Martin, V., Supanitsky, A., Echeverria, P.C., Litwin, S., Tanos, T., De Roodt, A.R., Guarnera, E.A., Angel, S.O., 2004. Recombinant GRA4 or ROP2 protein combined with alum or the gra4 gene provides partial protection in chronic murine models of toxoplasmosis. Clin. Diagn. Lab. Immunol. 11 (4), 704-710.

Miller, C., Quinn, H., Ryce, C., Reichel, M.P., Ellis, J.T., 2005. Reduction in transplacental transmission of *Neospora caninum* in outbred mice by vaccination. Int. J. Parasitol. 35 (7), 821-828.

Morrison, D.A., 2002. How to improve statistical analysis in parasitology research publications. Int. J. Parasitol. 32 (8), 1065-1070.

Mutwiri, G.K., Nichani, A.K., Babiuk, S., Babiuk, L.A., 2004. Strategies for enhancing the immunostimulatory effects of CpG oligodeoxynucleotides. J. Control. Release 97 (1), 1-17.

Near, K.A., Stowers, A.W., Jankovic, D., Kaslow, D.C., 2002. Improved immunogenicity and efficacy of the recombinant 19-kilodalton merozoite surface protein 1 by the addition of oligodeoxynucleotide and aluminum hydroxide gel in a murine malaria vaccine model. Infect. Immun. 70 (2), 692-701.

Pereira Garcia-Melo, D., Regidor-Cerrillo, J., Collantes-Fernandez, E., Aguado-Martinez, A., Del Pozo, I., Minguijon, E., Gomez-Bautista, M., Aduriz, G., Ortega-Mora, L.M., 2010. Pathogenic characterization in mice of *Neospora caninum* isolates obtained from asymptomatic calves. Parasitology 137 (7), 1057-1068.

Perez-Zaballos, F.J., Ortega-Mora, L.M., Alvarez-Garcia, G., Collantes-Fernandez, E., Navarro-Lozano, V., Garcia-Villada, L., Costas, E., 2005. Adaptation of *Neospora caninum* isolates to cell-culture changes: an argument in favor of its clonal population structure. J. Parasitol. 91 (3), 507-510.

Petersen, E., Nielsen, H.V., Christiansen, L., Spenter, J., 1998. Immunization with E. coli produced recombinant *T. gondii* SAG1 with alum as adjuvant protect mice against lethal infection with *Toxoplasma gondii*. Vaccine 16 (13), 1283-1289.

Pinitkiatisakul, S., Mattsson, J.G., Lunden, A., 2008. Quantitative analysis of parasite DNA in the blood of immunized and naive mice after infection with *Neospora caninum*. Parasitology 135 (2), 175-182.

Ramamoorthy, S., Lindsay, D.S., Schurig, G.G., Boyle, S.M., Duncan, R.B., Vemulapalli, R., Sriranganathan, N., 2006. Vaccination with gamma-irradiated *Neospora caninum* tachyzoites protects mice against acute challenge with *N. caninum*. J. Eukaryot. Microbiol. 53 (2), 151-156.

Ribeiro, D.P., Freitas, M.M., Cardoso, M.R., Pajuaba, A.C., Silva, N.M., Mineo, T.W., Silva, J.S., Mineo, J.R., Silva, D.A., 2009. CpG-ODN combined with *Neospora*

caninum lysate, but not with excreted-secreted antigen, enhances protection against infection in mice. Vaccine 27 (19), 2570-2579.

Rivera, E., Ekholm Pettersson, F., Inganas, M., Paulie, S., Gronvik, K.O., 2005. The Rb1 fraction of ginseng elicits a balanced Th1 and Th2 immune response. Vaccine 23 (46-47), 5411-5419.

Rivera, E., Hu, S., Concha, C., 2003. Ginseng and aluminium hydroxide act synergistically as vaccine adjuvants. Vaccine 21 (11-12), 1149-1157.

Rojo-Montejo, S., Collantes-Fernandez, E., Blanco-Murcia, J., Rodriguez-Bertos, A., Risco-Castillo, V., Ortega-Mora, L.M., 2009a. Experimental infection with a low virulence isolate of *Neospora caninum* at 70 days gestation in cattle did not result in foetopathy. Vet. Res. 40 (5), 49.

Rojo-Montejo, S., Collantes-Fernandez, E., Regidor-Cerrillo, J., Alvarez-Garcia, G., Marugan-Hernandez, V., Pedraza-Diaz, S., Blanco-Murcia, J., Prenafeta, A., Ortega-Mora, L.M., 2009b. Isolation and characterization of a bovine isolate of *Neospora caninum* with low virulence. Vet. Parasitol. 159 (1), 7-16.

Song, X., Hu, S., 2009. Adjuvant activities of saponins from traditional Chinese medicinal herbs. Vaccine 27 (36), 4883-4890.

Staska, L.M., McGuire, T.C., Davies, C.J., Lewin, H.A., Baszler, T.V., 2003. *Neospora caninum*-infected cattle develop parasite-specific CD4+ cytotoxic T lymphocytes. Infect. Immun. 71 (6), 3272-3279.

Su, Z., Tam, M.F., Jankovic, D., Stevenson, M.M., 2003. Vaccination with novel immunostimulatory adjuvants against blood-stage malaria in mice. Infect. Immun. 71 (9), 5178-5187.

Sun, J., Hu, S., Song, X., 2007. Adjuvant effects of protopanaxadiol and protopanaxatriol saponins from ginseng roots on the immune responses to ovalbumin in mice. Vaccine 25 (6), 1114-1120.

Sun, J., Song, X., Hu, S., 2008. Ginsenoside Rg1 and aluminum hydroxide synergistically promote immune responses to ovalbumin in BALB/c mice. Clin. Vaccine Immunol. 15 (2), 303-307.

1 Tonui, W.K., Mejia, J.S., Hochberg, L., Mbow, M.L., Ryan, J.R., Chan, A.S., Martin,
2 S.K., Titus, R.G., 2004. Immunization with *Leishmania major* exogenous antigens
3 protects susceptible BALB/c mice against challenge infection with *L. major*. Infect.
4 Immun. 72 (10), 5654-5661.

5 Tritto, E., Mosca, F., De Gregorio, E., 2009. Mechanism of action of licensed vaccine
6 adjuvants. Vaccine 27 (25-26), 3331-3334.

7 Williams, D.J., Guy, C.S., Smith, R.F., Ellis, J., Bjorkman, C., Reichel, M.P., Trees,
8 A.J., 2007. Immunization of cattle with live tachyzoites of *Neospora caninum* confers
9 protection against fetal death. Infect. Immun. 75 (3), 1343-1348.

10

Tables

Table 1. Summary of group characteristics.

Groups	Adjuvant	Killed tachyzoites dose	Challenge dose (Nc-1)
1	A	10^5	10^6
2	A	5×10^5	10^6
3	A	10^6	10^6
4	A	PBS	10^6
5	B	10^5	10^6
6	B	5×10^5	10^6
7	B	10^6	10^6
8	B	PBS	10^6
9	C	10^5	10^6
10	C	5×10^5	10^6
11	C	10^6	10^6
12	C	PBS	10^6
13	PBS	10^5	10^6
14	PBS	5×10^5	10^6
15	PBS	10^6	10^6
16	PBS	PBS	10^6
17	PBS	PBS	PBS

Table 2. Detection of *N. caninum* DNA by nested-PCR in blood, lungs and brain on days 1-5 (a) and days 14-30 post-challenge (b).

Groups	Blood		Lung		Brain	
	a	**b**	**a**	**b**	**a**	**b**
1 (A/10^5)	9/10[a]	1/10	5/10	0/10	1/10	2/10
2 (A/5 × 10^5)	7/10	0/10	5/10	0/10	1/10	0/10
3 (A/10^6)	8/10	0/10	6/10	0/10	1/10	3/10
4 (A/PBS)	8/10	0/10	5/10	1/10	0/10	5/10
5 (B/10^5)	8/10	1/10	5/10	2/10	0/10	4/10
6 (B/5 × 10^5)	8/10	0/10	3/10	2/10	0/10	3/10
7 (B/10^6)	7/10	0/10	4/10	2/10	1/10	1/10
8 (B/PBS)	8/10	0/10	4/10	0/10	0/10	6/10
9 (C/10^5)	5/10	1/10	4/10	3/10	0/10	7/10
10 (C/5 × 10^5)	2/10	1/10	6/10	3/10	1/10	7/10
11 (C/10^6)	4/10	1/10	2/10	5/10	0/10	8/10
12 (C/PBS)	8/10	0/10	4/10	0/10	0/10	4/10
13 (PBS/10^5)	10/10	1/10	7/10	2/10	2/10	6/10
14 (PBS/5 × 10^5)	9/10	0/10	5/10	4/10	0/10	8/10
15 (PBS/10^6)	7/10	0/10	3/10	4/10	1/10	5/10
16 (non-immunized/ challenged control)	8/10	0/10	5/10	1/10	1/10	5/10
17 (non-immunized/ non-challenged control)	0/10	0/10	0/10	0/10	0/10	0/10

[a]Fractions represent number of mice positive by nested-PCR/ number of mice tested.

Figure legends

Figure 1. Box-plot and whiskers graph represents the lower, upper quartiles, median and minimum-maximum of the parasite burden (number of parasites per μg of host DNA) in (A) blood on days 1-5 post-challenge, and (B) lungs and (C) the brain on days 14-30 post-challenge. Mice were s.c. immunized with 10^5 (groups 1, 5, 9 and 13), $5x10^5$ (groups 2, 6, 10 and 14) or 10^6 (groups 3, 7, 11 and 15) inactivated whole tachyzoites or no antigen (groups 4, 8 and 12) incorporated into adjuvant A (water-in-oil emulsion), B (aluminum hydroxide-CpG), C (aluminum hydroxide-ginseng extract) or PBS. Taking into account that the *N. caninum* detection limit by real-time PCR is 10^{-1} parasites (Collantes-Fernández et al., 2002), all positive samples had ≥ 0.1 parasites. Negative samples (0 parasites) were represented in log scale as < 0.1 parasites.

Figure 2. Box-plot and whiskers graph represents the lower, upper quartiles, median and minimum-maximum of the lesion severity score (nil = 0, mild = 1, moderate=2, and severe = 3) in the brain on days 14-30 post-challenge. Mice were s.c. immunized with 10^5 (groups 1, 5, 9 and 13), $5x10^5$ (groups 2, 6, 10 and 14) or 10^6 (groups 3, 7, 11 and 15) inactivated whole tachyzoites or no antigen (groups 4, 8 and 12) incorporated into adjuvant A (water-in-oil emulsion), B (aluminum hydroxide-CpG), C (aluminum hydroxide-ginseng extract) or PBS.

Figure 3. Levels of anti-*N. caninum*-specific IgG2a and IgG1 in sera of BALB/c mice immunized subcutaneously twice at three-week intervals. Blood samples were collected from the mice 1-5 days (A) and 14-30 days after the challenge (B). The specific IgG2a and IgG1 levels were assessed using ELISA. Bars represents the average absorbance value at 405 nm and standard error bars represent the standard deviation.

Figure 1A

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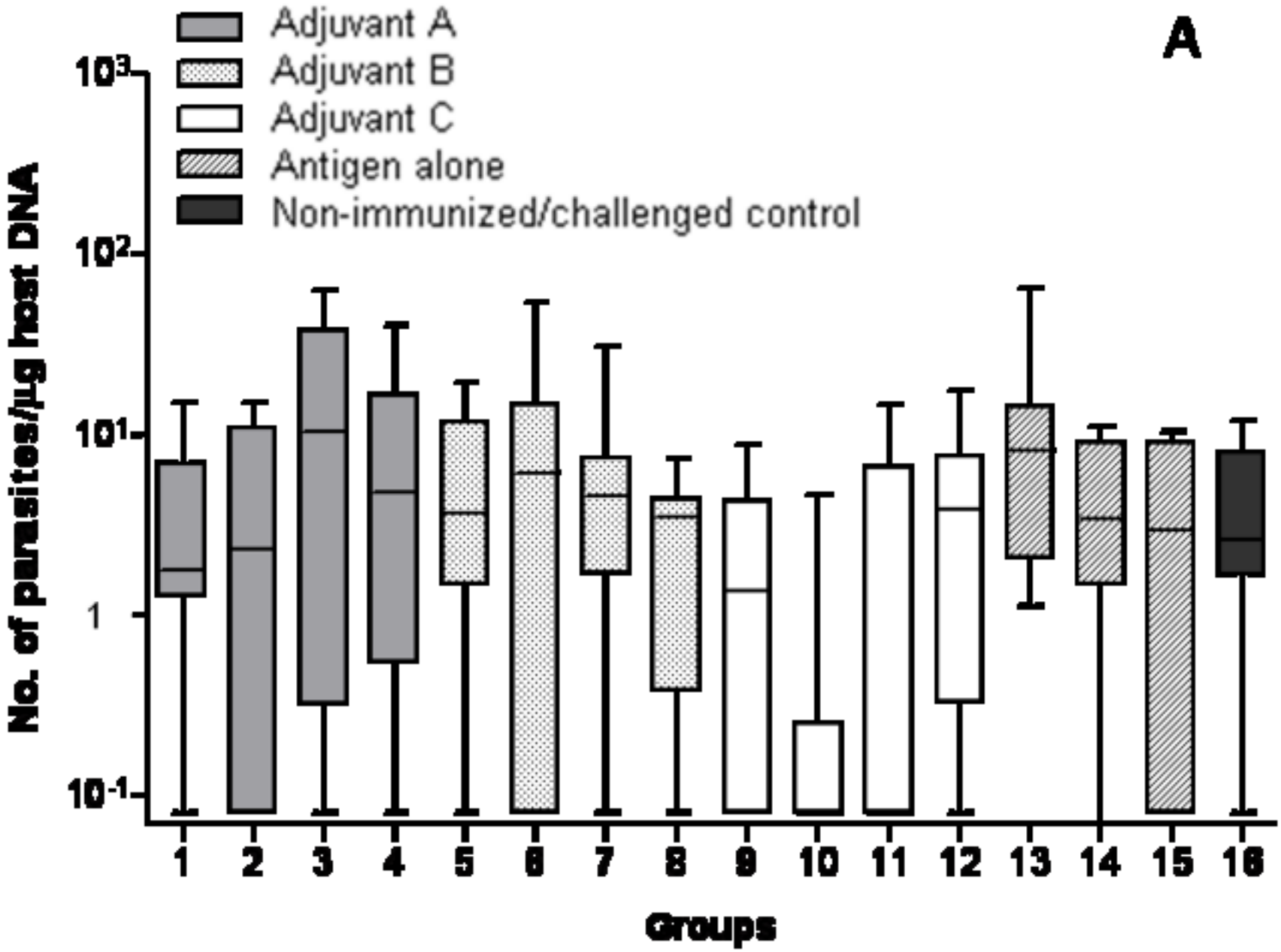

Figure 1B

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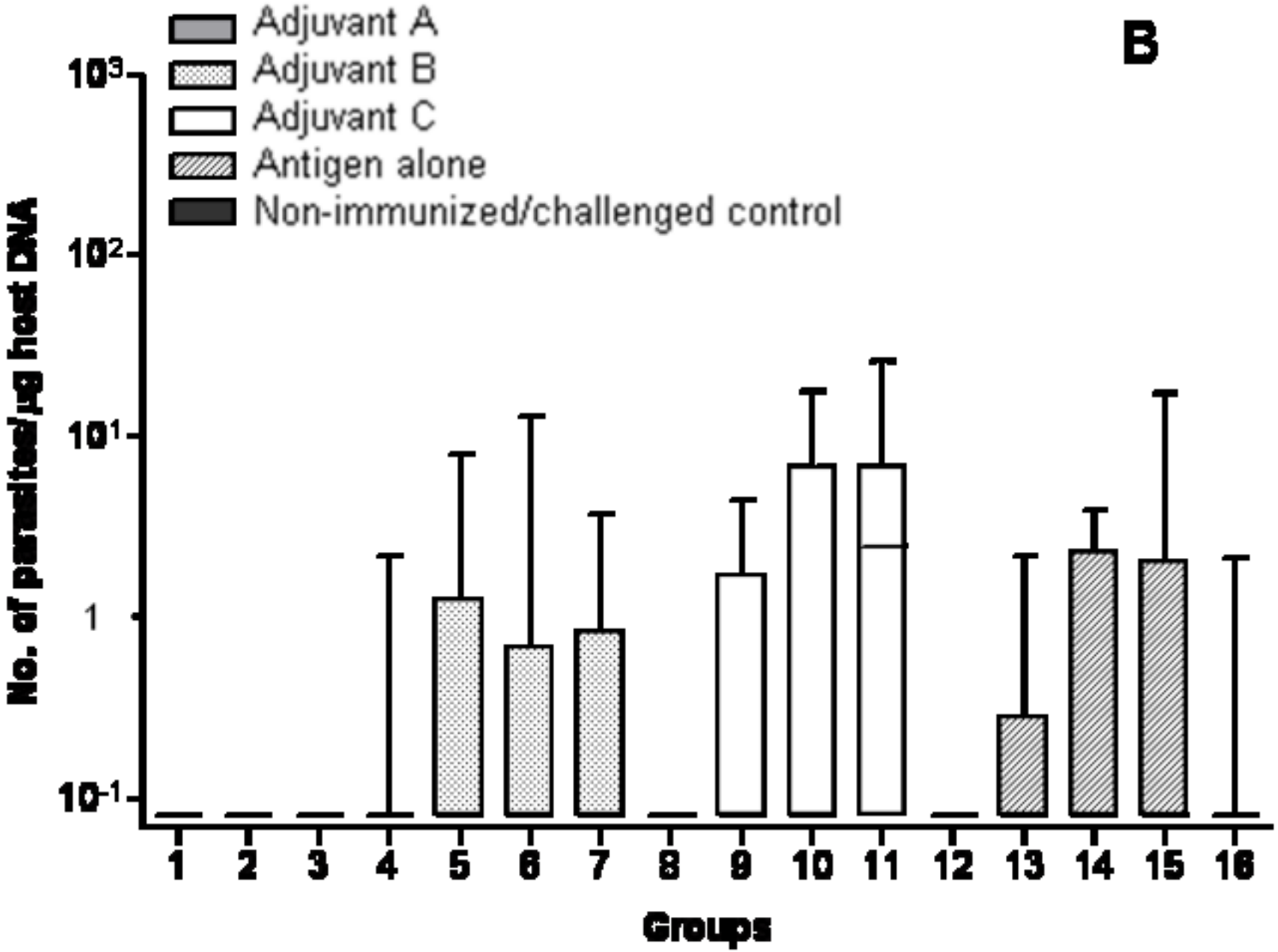

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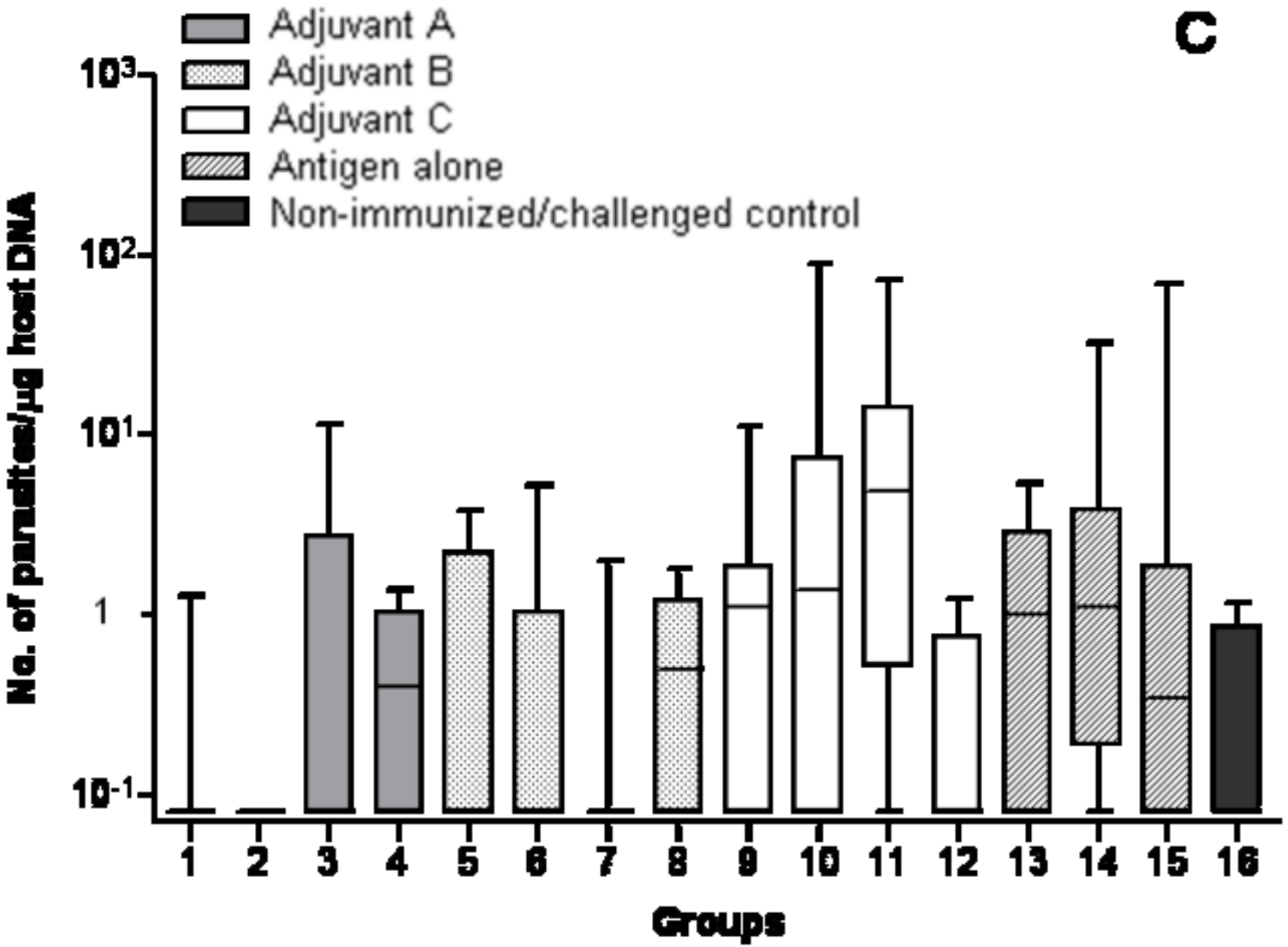

Figure 2

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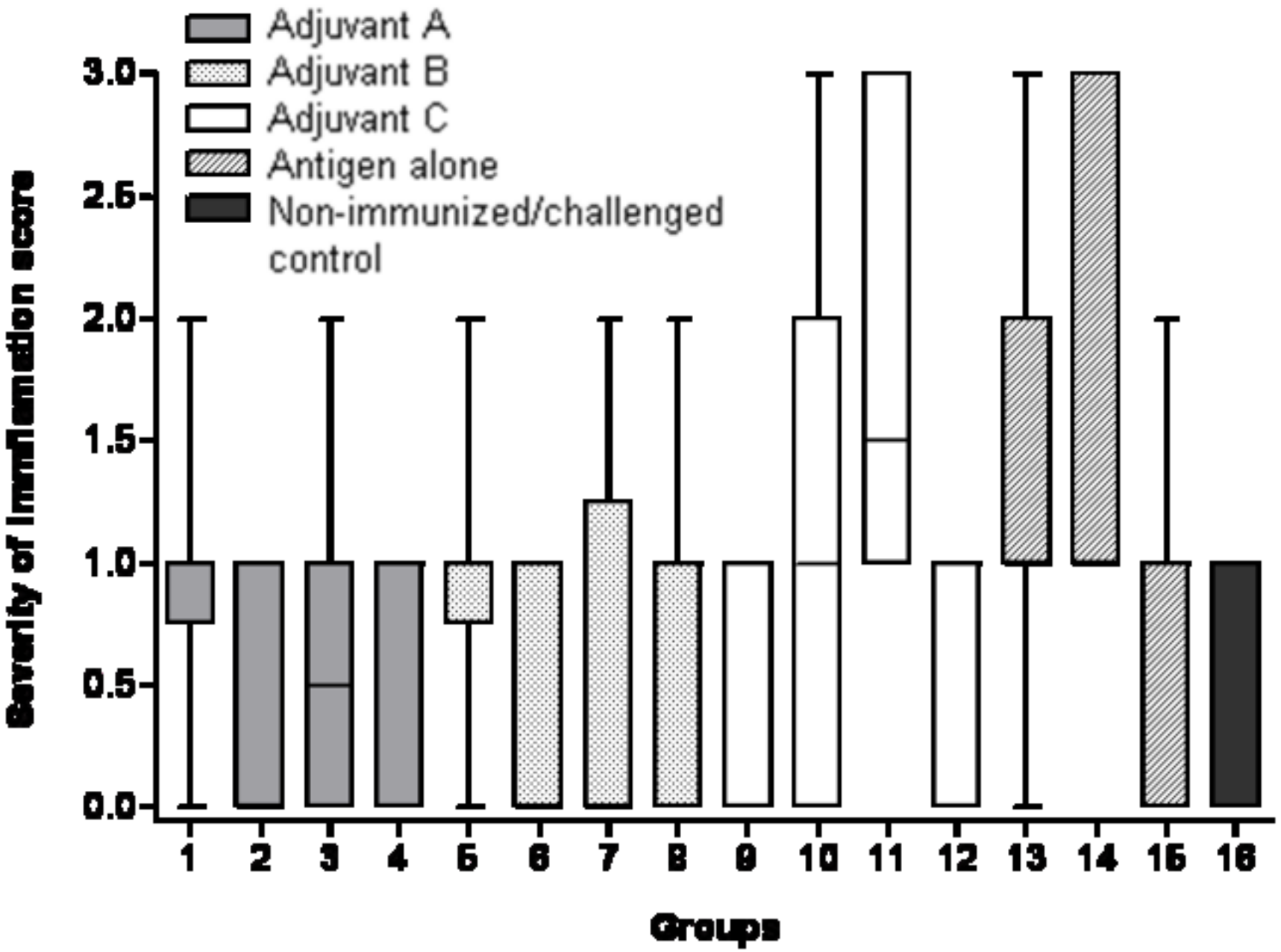

Figure 3A

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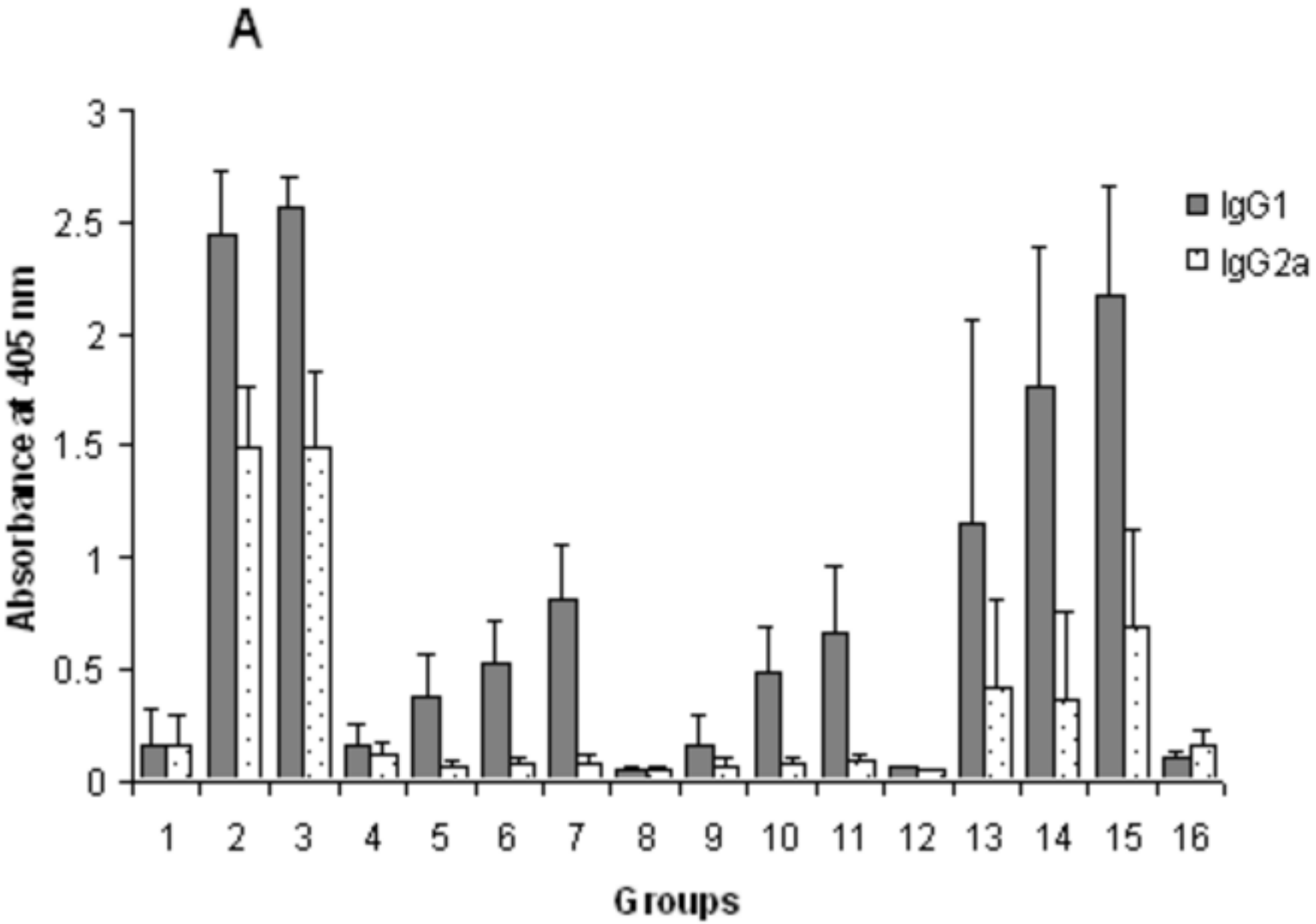

Figure 3B

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