**Research Article**

**Title** Comparison of the Immunogenicity of Different Proportions of Chitosan Adjuvant in Attenuated *Neospora caninum* Vaccine in BALB/c Mice



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**Abstract**

Neosporosis is a neuromuscular infectious disease in dogs caused by the protozoan *N. caninum*. This disease is globally distributed; dogs and canines are the definitive hosts, while a wide range of animals serve as intermediate hosts. Therefore, this study aimed to compare the immunogenicity of different proportions of chitosan adjuvant in an attenuated *N. caninum* vaccine in BALB/c mice. Twenty-eight BALB/c mice were divided into four groups of seven. Groups 1, 2, and 3 were subcutaneously immunized with attenuated tachyzoites of *N. caninum*

strain Nc-1 combined with micronized chitosan adjuvant at concentrations of 10%, 50%, and 90%, respectively. The fourth group served as the control. All groups were monitored daily over a three-week period. Following sample collection, antibody titters were quantified using ELISA, and cellular immune responses were evaluated by measuring IFN-γ levels with a commercially available assay kit. According to ELISA results, the highest antibody levels were observed in the group immunized with the attenuated *N. caninum* strain plus 90% chitosan adjuvant, showing a significant difference compared to the control and other groups ($P < 0.05$). Measurement of IFN-γ revealed that the strongest cellular immune response was observed in the group immunized with 90% chitosan adjuvant, with significant differences compared to the control and other groups ($P < 0.05$). Based on these findings and the considerable effect of chitosan adjuvant on both antibody titers and cellular immunity, the use of this adjuvant is recommended in the development of *Neospora* vaccines.



## Introduction

*N. caninum* is a protozoan parasite transmitted transplacentally, with a wide range of intermediate hosts and canines as definitive hosts. In many countries, it is a leading cause of abortion in cattle and a significant cause of neuromuscular paralysis in dogs [1]. Cattle can be infected congenitally through placental transmission or postnatally by ingesting oocysts shed by the definitive hosts. Currently, there is no effective drug to treat infected cattle; therefore, prevention relies mainly on management and hygiene practices [2]. Developing an effective vaccine would greatly aid in controlling this disease.

Chitosan is a natural polysaccharide derived from the partial deacetylation of chitin. It possesses polymeric properties that vary according to its degree of deacetylation, molecular weight, viscosity, and the number of amino groups. These features enable chitosan to chemically

interact with anionic systems, resulting in changes to its physicochemical properties. Consequently, chitosan exhibits excellent biological properties, including biodegradability in the human body, as well as immunological, antibacterial, and wound healing activities [3,4]. This study aimed to compare the immunogenicity of different proportions of chitosan adjuvant in an attenuated *N. caninum* vaccine in BALB/c mice.



**Results**

Evaluation of Vero cell line culture and passage:

Visual inspection of the culture flasks indicated clarity and transparency, suggesting no microbial contamination. In the absence of contamination, the culture medium remained clear. Microscopic observation revealed that Vero cells retained their characteristic regular polyhedral morphology. Tachyzoite release and cell destruction occurred within 4 to 7 days post-inoculation. The harvested tachyzoites were then utilized for immunization in mice.

Assessment of microbial contamination in vaccine preparations:

Cell culture monitoring for 48 hours showed no signs of microbial contamination in the prepared vaccine samples. Extended observation over two weeks confirmed the absence of *Mycoplasma* contamination in the cell lines used for vaccine production.

Immunization outcomes in experimental mice:

To evaluate the immunogenic potential of the experimental formulations, both humoral and cellular immune responses were analyzed using ELISA and IFN-γ assays. Statistical differences among the groups were assessed using one-way ANOVA, followed by post-hoc pairwise comparisons with adjusted P-values. A significance threshold of $P < 0.05$ was applied.

The ELISA results demonstrated that the group immunized with the attenuated *N. caninum* strain combined with 90% chitosan adjuvant (G4) exhibited the highest antibody titers. In contrast, Group G3 displayed the lowest humoral immune responses. Comparisons indicated

that G4 induced significantly stronger antibody responses compared to all other groups (vs. G1, P = 0.0002; vs. G2, P = 0.0002; vs. G3, P < 0.0001). No significant difference was observed between Groups G1 and G2 (P = 0.9496), while G1 showed higher antibody titers than G3 (P = 0.0474). Similarly, G2 also elicited stronger antibody responses than G3 (P = 0.0320). Analysis of IFN-γ levels further confirmed these trends. Group G4 induced the strongest cellular immune responses, which were significantly higher than those of G1 (P = 0.0003), G2 (P = 0.0002), and G3 (P < 0.0001). No significant difference was detected between G1 and G2 (P = 0.3333). In contrast, Group G3 exhibited a markedly weaker cellular response compared to both G1 (P = 0.0005) and G2 (P = 0.0008).

Overall, these findings demonstrate that the 90% chitosan adjuvant formulation (G4) significantly enhanced both humoral and cellular immune responses compared to all other groups, highlighting its superior immunostimulatory efficacy. Group G3 produced the weakest responses, while Groups G1 and G2 displayed intermediate and comparable levels of immunity (Table 1.).

**Table 1.**
The analyses were obtained using one-way ANOVA test

| **Comparisons** | **Mean Diff.** | | **95.00% CI of diff.** | | **Adjusted P Value** | |
|---|---|---|---|---|---|---|
| **Groups** | ELISA | Gamma interferon | ELISA | Gamma interferon | ELISA | Gamma interferon |
| G1 vs. G2 | 0.03550 | -0.04500 | -0.2419 to 0.3129 | -0.1383 to 0.04828 | 0.9496 | 0.3333 |
| G1 vs. G3 | -0.2820 | -0.3300 | -0.5594 to -0.004647 | -0.4233 to -0.2367 | 0.0474 | 0.0005 |
| G1 vs. G4 | 1.262 | 0.3650 | 0.9846 to 1.539 | 0.2717 to 0.4583 | 0.0002 | 0.0003 |
| G2 vs. G3 | -0.3175 | -0.2850 | -0.5949 to -0.04015 | -0.3783 to -0.1917 | 0.0320 | 0.0008 |
| G2 vs. G4 | 1.227 | 0.4100 | 0.9491 to 1.504 | 0.3167 to 0.5033 | 0.0002 | 0.0002 |
| G3 vs. G4 | 1.544 | 0.6950 | 1.267 to 1.821 | 0.6017 to 0.7883 | 0.0001 | <0.0001 |

## Discussion

A review of the literature revealed that limited studies have investigated the effects of various adjuvants on antibody levels and cellular immunity titers, particularly in the context of *N. caninum* vaccines. Consistent with the findings of this study, the use of chitosan as an adjuvant significantly increased antibody levels in the tested samples. Moreover, chitosan exhibited a notable and statistically significant enhancement of cellular immunity. Adjuvants are critical components of vaccines, as they not only enhance immunogenicity but are also safe for use in both humans and animals [7].

In this study, the highest antibody titers were observed in the group immunized with the attenuated *N. caninum* strain combined with chitosan adjuvant. The results highlight the significant impact of chitosan on cellular immunity as well. Previous research has demonstrated that adjuvants can induce long-term humoral immunity, a valuable advantage when developing vaccines. However, it is important to note that humoral immunity alone is often insufficient to protect against parasitic infections, which require robust cellular immune responses.

For example, Guo et al. (2018) investigated the immunomodulatory effect of chitosan adjuvant in combination with a *Toxoplasma gondii* protein vaccine in mice. They reported significant increases in IFN-γ, interleukin-4, and interleukin-10 levels, concluding that chitosan could serve as an effective carrier system for toxoplasmosis vaccines [8]. These findings align well with the results of the current study, emphasizing the potent immunostimulatory properties of chitosan. While previous studies often utilized chitosan nanoparticles, this study employed micronized chitosan, which nonetheless generated a strong antibody response and cellular immunity.

Cellular immunity plays a crucial role in protective immunity against neosporosis, primarily through the production and secretion of cytokines such as IFN-γ. This cytokine, produced by T cells, is essential for controlling *N. caninum* infection by activating intracellular mechanisms

that eliminate the parasite. Studies have also shown that mice, as experimental models, have limited endogenous IFN-γ production, which can be significantly enhanced by adjuvants such as chitosan [9]. Consistent with these reports, this study demonstrated that chitosan adjuvant significantly increased IFN-γ production, thereby boosting cellular immunity. Additionally, chitosan has been shown to promote a Th2 immune response, reflected in the elevated antibody titers observed in immunized mice compared to controls. These findings collectively underscore the importance of chitosan as a potent adjuvant capable of stimulating both humoral and cellular immune responses. Overall, the results of this study demonstrate a positive and significant effect of chitosan adjuvant on the immune response against *N. caninum*. ELISA tests revealed that the group immunized with attenuated *N. caninum* combined with 90% chitosan adjuvant exhibited the highest antibody levels, significantly greater than those of the control and other groups ($P < 0.05$). Similarly, IFN-γ assays confirmed that this group mounted the strongest cellular immune response ($P < 0.05$). Based on these findings, the use of chitosan as an adjuvant is strongly recommended in the development of effective *Neospora* vaccines to enhance both humoral and cellular immunity.



**Materials and Methods**

Ethical approval:

All procedures in this experiment were approved by the Institutional Animal Care and Use Committee of Karaj Islamic Azad University with Code Ethics ID (IR.IAU.K.REC.1402.43).

Parasite culture:

Tachyzoites of *N. caninum* isolate NC-1 was obtained from the Razi Institute, Shiraz branch. Parasites were cultured on a monolayer of Vero cells in RPMI 1640 medium supplemented with 2% fetal calf serum, penicillin (10,000 U/ml), streptomycin (100 µg/ml), and amphotericin B (25 µg/ml) at 37°C in a 5% $CO_2$ incubator. Five days post-culture, tachyzoites were harvested

by scraping the monolayer cells, centrifuged, and the inoculum concentration was adjusted using a Neubauer hemocytometer and diluted with RPMI medium as needed [5].

In vitro tests:

Passaging adherent Vero cells:

*N. caninum* was cultured on Vero cell monolayers prepared 24 hours prior in 25 $cm^2$ flasks with DMEM supplemented with 2% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (25 µg/ml). Cultures were maintained at 37°C with 5% $CO_2$ and monitored daily by inverted microscopy for cell destruction. When 80–90% of the Vero cells were destroyed (typically within 3–5 days), the supernatant was collected, mixed with PBS, and centrifuged at 1000 rpm for 5 minutes. Tachyzoites were counted using a Neubauer hemocytometer.

Passaging semi-adherent J774 cells without trypsin:

J774 cells (BALB/c mouse macrophage-like cells) were thawed under sterile conditions and cultured in 25 $cm^2$ flasks with DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), gentamycin (50 µg/ml), and amphotericin B (25 µg/ml). Cultures were incubated at 37°C with 5% $CO_2$ and monitored daily. For passaging, flasks were washed with PBS, cells were scraped, collected into 15 ml Falcon tubes, and centrifuged at 1000 × g for 5 minutes. The pellet was resuspended in fresh culture medium and transferred back to flasks for continued incubation.

Parasite preparation and attenuation by successive passages on J774 cells:

To prepare the attenuated strain for vaccination, the culture supernatant containing tachyzoites was centrifuged at 500 rpm for 10 minutes. The pellet was washed with PBS under sterile

conditions. Live attenuated *Neospora* tachyzoites, collected from J774 cultures, were counted and viability assessed by trypan blue staining. The inoculum dose was adjusted for injection into mice.

*Mycoplasma* contamination testing:

Due to the risk of *Mycoplasma* contamination in cell cultures, all cultures were tested using PPLO broth and agar media. Samples were incubated at 37°C for 24–48 hours in PPLO broth (pH 7.6–8), then 0.2 ml of each sample was plated on PPLO agar (3.5%, pH 6.7–8) and incubated for 21 days at 37°C. Cultures were monitored for colony growth and pH changes. *Escherichia coli* was used as a positive control on blood agar to verify sterility.

Study animals and vaccination:

Twenty-eight BALB/c mice were randomly divided into four groups of seven. Groups 1, 2, and 3 were subcutaneously injected with $2\times10^6$ attenuated *N. caninum* tachyzoites (strain Nc-1) combined with micronized chitosan adjuvant at concentrations of 10%, 50%, and 90%, respectively. The fourth group served as the negative control and did not receive any vaccine formulation or adjuvant. Animals were observed daily for a period of three weeks to monitor their general health status. At the end of the experimental period, blood samples were collected from all mice, and sera were separated and stored at –20 °C until subsequent immunological analyses.

Antigen preparation and ELISA test:

*Neospora* antigen was prepared by sonicating $2\times10^9$ tachyzoites in 1 ml of phenylmethylsulphonyl fluoride (2 mM) on ice. The mixture was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was used as the antigen. ELISA plates were coated with

100 µl antigen and bicarbonate buffer, incubated at 4°C for 24 hours, then blocked with 3% bovine serum albumin in PBS-Tween (0.05%) for 2 hours at room temperature. After washing, mouse serum samples diluted 1:100 were added and incubated at 37°C for 1 hour. Plates were washed, and 100 µl of anti-mouse HRP-conjugated IgG (diluted 1:2000) was added and incubated at 37°C for 1 hour. After washing, 100 µl of OPD substrate was added and incubated in the dark for 15 minutes, then the reaction was stopped with 100 µl of 12.5% sulfuric acid. Absorbance was read at 450 nm [6].

Gamma interferon (IFN-γ) assay:

Gamma interferon levels were quantified using a commercial mouse IFN-γ ELISA kit (Invitrogen™ Mouse IFN-γ ELISA Kit, Thermo Fisher Scientific, Waltham, MA, USA) based on monoclonal antibodies, following the manufacturer's protocol. The required sample volume was determined according to the Resource Equation Approach.

Data analysis:

Statistical analysis was performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was applied, and *P-values* less than 0.05 were considered statistically significant.

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# عنوان مقاله: مقایسه ایمنی‌زایی نسبت‌های مختلف آدجوانت کیتوزان در واکسن تضعیف‌شده *نئوسپورا کنینوم* در موش‌های BALB/c


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**خلاصه فارسی:** نئوسپوروزیس یک بیماری عفونی عصبی – عضلانی در سگ‌ها است که توسط تک‌یاخته *نئوسپورا کنینوم* ایجاد می‌شود. این بیماری به‌طور گسترده در جهان پراکنده است؛ سگ‌ها و سگ‌سانان میزبان نهایی هستند و میزبان‌های واسطه متنوعی نیز وجود دارند. بنابراین، هدف این مطالعه مقایسه ایمنی‌زایی نسبت‌های مختلف آدجوانت کیتوزان در واکسن تضعیف‌شده *نئوسپورا کنینوم* در موش‌های BALB/c بود. بیست و هشت موش BALB/c به چهار گروه ۷تایی تقسیم شدند. گروه‌های ۱، ۲ و ۳ به‌صورت زیرجلدی با تاکیزوئیت‌های تضعیف‌شده *نئوسپورا کانینوم* سویه Nc-1 همراه با آدجوانت کیتوزان میکرونیزه شده به نسبت‌های ۱۰٪، ۵۰٪ و ۹۰٪ ایمن‌سازی شدند. گروه چهارم به عنوان کنترل در نظر گرفته شد. تمام گروه‌ها به مدت سه هفته روزانه تحت نظر قرار گرفتند. پس از نمونه‌برداری، تیتر آنتی‌بادی‌ها توسط ELISA اندازه‌گیری و ایمنی سلولی با سنجش میزان گاما اینترفرون با کیت تجاری ارزیابی شد. نتایج ELISA ، بالاترین سطح آنتی‌بادی در گروه ایمن‌شده با واکسن تضعیف‌شده به همراه ۹۰٪ آدجوانت کیتوزان را نشان داد که اختلاف معنی‌داری نسبت به گروه کنترل و سایر گروه‌ها داشت ($P < 0.05$). در اندازه گیری گاما اینترفرون نیز، قوی‌ترین پاسخ ایمنی سلولی مربوط به گروه ۹۰٪ کیتوزان بود که نسبت به گروه کنترل و سایر گروه‌ها تفاوت معنی‌دار داشت .($P < 0.05$) بر اساس این نتایج و اثر قابل توجه آدجوانت کیتوزان بر تیتر آنتی‌بادی‌ها و ایمنی سلولی، استفاده از این آدجوانت در توسعه واکسن‌های *نئوسپورا* توصیه می‌شود.



**واژگان کلیدی:** واکسن، *نئوسپورا کنینوم*، آدجوانت، کیتوزان.