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RESEARCH ARTICLE

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Comparison of the Immunogenicity of Different Proportions of Chitosan Adjuvant in an 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; canines are the definitive hosts, while a wide range of animals serve as intermediate hosts. 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 (n = 7 per group). 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. Group 4 served as the negative control and did not receive any vaccine formulation or adjuvant. All groups were monitored daily over a three-week period. Following sample collection, antibody levels were quantified using ELISA, and cellular immune responses were evaluated by measuring IFN-γ levels with a commercial assay kit. According to ELISA results, the highest antibody levels were observed in the group immunized with the attenuated *N. caninum* strain containing 90% chitosan adjuvant, showing significantly higher antibody levels compared to the control and other groups ($p < 0.05$). Similarly, IFN-γ analysis revealed that the strongest IFN-γ-associated immune response was observed in the group immunized with 90% chitosan adjuvant, with significant differences compared to the control and other groups ($p < 0.05$). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Based on these findings and the considerable effect of chitosan adjuvant on both antibody levels and IFN-γ-associated cellular immunity, these findings support further investigation of chitosan-containing formulations as potential adjuvants for Neospora vaccine development.

Keywords

Neospora caninum, Chitosan, Vaccine adjuvant, Immunogenicity, BALB/c mice, IFN-γ

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Abbreviations

ANOVA: Analysis of Variance
(BALB/c): Bagg Albino laboratory mouse strain
DMEM: Dulbecco's Modified Eagle Medium
ELISA: Enzyme-Linked Immunosorbent Assay

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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].

Chitosan has been extensively studied as a vaccine adjuvant due to its biocompatibility, biodegradability, and immunostimulatory properties. Previous studies have demonstrated that chitosan-based formulations can enhance both humoral and cellular immune responses against various pathogens. For instance, Guo et al. (2018) demonstrated that a chitosan microsphere vaccine significantly increased IFN-γ, IL-4, and IL-10 levels in mice immunized against Toxoplasma gondii [8]. However, limited research has been conducted on the use of chitosan as an adjuvant in Neospora caninum vaccines. While attenuated *N. caninum* vaccines have shown promise in inducing protective immunity [ref], the optimal adjuvant formulation for enhancing their immunogenicity remains to be established.

To our knowledge, no study has systematically compared the immunogenicity of different proportions of micronized chitosan when combined with an attenuated *N. caninum* vaccine. The concentrations of 10%, 50%, and 90% were selected based on a preliminary dose-finding study (data not shown) that evaluated a range of chitosan concentrations (5–95%) for compatibility with the vaccine formulation and absence of local toxicity. These three concentrations were chosen to represent a low (10%), medium (50%), and high (90%) proportion of chitosan in the final vaccine formulation, allowing for a systematic evaluation of the dose-response relationship.

We hypothesized that increasing the concentration of micronized chitosan adjuvant would enhance both humoral and cellular immune responses to the attenuated *N. caninum* vaccine in a dose dependent manner. Therefore, this study aimed to compare the immunogenicity of different proportions of chitosan adjuvant in an attenuated *N. caninum* vaccine in BALB/c mice.

Abbreviations-Cont'd

FBS: Fetal Bovine Serum
IFN-γ: Interferon-gamma
J774: Murine macrophage-like cell line OD: Optical Density
OPD: o-Phenylenediamine
PBS: Phosphate-Buffered Saline
PPLO: Pleuropneumonia-like Organism
RPMI: Roswell Park Memorial Institute medium Vero: African Green Monkey kidney cell line

Results

Evaluation of Vero cell line culture and passage

Visual inspection of the culture flasks indicated clear and transparent medium, suggesting no microbial contamination. Microscopic examination showed that Vero cells preserved their characteristic regular polyhedral morphology. Followin g inoculation, tachyzoite release accompanied by progressive cell destruction was observed within 4 to 7 days post-inoculation. The harvested tachyzoites obtained at peak release (yield range: 5.2×10^7 to 8.1×10^7 per flask, viability >95% by trypan blue exclusion) were subsequently used for immunization.

Assessment of microbial contamination in vaccine preparations

Cell culture monitoring over 48 hours showed no evidence of microbial contamination in the prepared vaccine formulations. Extended observation for 21 days 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.

The ELISA results demonstrated that the group immunized with the attenuated *N. caninum* strain combined with 90% chitosan adjuvant (G3) exhibited the highest antibody levels. In contrast, Group G4 (control) displayed the lowest responses. Comparisons indicated that G3 induced significantly higher antibody levels compared to all other groups (vs. G1, $p = 0.0002$; vs. G2, $p = 0.0002$; vs. G4, $p < 0.0001$). No

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

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

Table 1.
Pairwise comparisons of ELISA (OD450) and IFN-γ (pg/mL) responses between experimental groups.

Comparisons Groups	Mean Diff.		95.00% CI of diff.		Adjusted P Value	
	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

Data are presented as mean ± SD (n=7 per group). Group labels: G1 = 10% chitosan; G2 = 50% chitosan; G3 = 90% chitosan; G4 = negative control. P values adjusted for multiple comparisons using Tukey's post-hoc test.

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 formulation containing chitosan was associated with significantly higher antibody levels in the tested samples. In addition, the chitosan-containing formulation exhibited a notable and statistically significant enhancement of IFN-γ-associated immune responses. Adjuvants are critical components of vaccines, as they not only enhance immunogenicity but are also safe for use in both humans and veterinary applications [7].

In the present study, the highest antibody levels were observed in the group immunized with the attenuated *N. caninum* strain combined with 90% chitosan adjuvant (G3). The results highlight the significant impact of the chitosan-containing formulation on IFN-γ-associated immune responses. Previous research has demonstrated that adjuvants can induce long-term humoral immunity, which is a key advantage in vaccine development. However, it is important to note that humoral immunity alone is often insufficient to protect against parasitic infections, which typically require robust cellular immune responses.

For instance, Guo et al. (2018) investigated the immunomodulatory effect of chitosan adjuvant in combination with a Toxoplasma gondii protein vaccine in mice. Their findings showed significant increases in IFN-γ, interleukin-4, and interleukin-10 levels, leading to the conclusion that chitosan may serve as an effective carrier system for toxoplasmosis vaccines [8]. While this is consistent with our findings in *N. caninum*, important differences between the two parasites (*T. gondii* vs. *N. caninum*), antigens, and vaccine formulations must be considered. Additionally, the present study used micronized chitosan rather than chitosan nanoparticles used in previous studies.

Cellular immunity plays a crucial role in protec-

tive 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 through the activation of intracellular parasite killing mechanisms. Studies have also shown that mice, as experimental models, have limited endogenous IFN-γ production, which can be significantly enhanced through the administration of adjuvants such as chitosan [9]. Consistent with these reports, the present study demonstrated that the chitosan-containing formulation significantly increased IFN-γ levels, thereby indicating enhanced Th1-associated cellular immune responses. Additionally, chitosan has been previously shown to promote Th2 immune responses in other studies (Singla & Chawla, 2001), which is consistent with the elevated antibody levels observed in the present study. However, the current study did not directly measure Th2-associated cytokines (e.g., IL-4, IL-10), and further studies are needed to confirm Th2 polarization.

These findings collectively underscore the importance of chitosan as a potent adjuvant capable of stimulating both humoral and IFN-γ-associated immune responses. Overall, the results of this study demonstrate a positive and significant effect of chitosan-containing formulations on the immune response against *N. caninum*. ELISA tests revealed that the group immunized with attenuated *N. caninum* vaccine combined with 90% chitosan adjuvant (G3) 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 IFN-γ-associated immune response ($p < 0.05$).

While our findings demonstrate that the 90% chitosan formulation significantly enhances immunogenicity (antibody and IFN-γ responses), these results do not establish protective efficacy. Challenge studies with *N. caninum* infection are needed to determine whether the observed immune responses translate to clinical protection. Therefore, we recommend further investigation of chitosan-containing formulations in protective efficacy studies.

Limitations

Several limitations of this study should be acknowledged:

1- The sample size (n=7 per group) was relatively small, and findings should be validated in larger studies.

2- No vaccine-only (without chitosan) or chitosan-only groups were included, limiting our ability to isolate the independent adjuvant effect of chitosan versus the vaccine antigen.

3- No challenge infection was performed, so the protective efficacy of the observed immune responses could not be evaluated.

4- Assessment was limited to ELISA and IFN-γ; direct cellular immune phenotyping (CD4/CD8, lymphocyte proliferation) was not performed.

5- The attenuated vaccine was validated by reduced virulence, but detailed characterization of attenuation stability was limited.

6- The study duration (three weeks post-immunization) does not address long-term immunogenicitted to 2×10^6 tachyzoites per mouse.

Materials and Methods

Declaration of Generative AI and AI-assisted technologies in the writing process

Artificial intelligence has been used to improve the grammar of the English text.

Ethical approval

All procedures in this experiment were approved by the Institutional Animal Care and Use Committee of Karaj Islamic Azad University under the ethical approval Code IIR. IAU.K.REC.1402.43. for continued incubation.

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 in PPLO broth (pH 7.6–8) at 37°C for 24–48 hours. Subsequently, 0.2 mL of each sample was inoculated onto PPLO agar (3.5%, pH 6.7–8) and incubated at 37°C for 21 days. Cultures were monitored for colony growth and pH changes. Escherichia coli (ATCC 25922) was used as a positive control on blood agar to verify that the culture medium supported bacterial growth and that the detection system was functional. Sterility of the vaccine preparations was confirmed by absence of bacterial growth on blood agar and absence of Mycoplasma growth on PPLO agar after 21 days.

Study animals and vaccination

A total of twenty-eight female BALB/c mice (6–8 weeks old, weight 18–22 g) were obtained from [source]. Animals were housed under standard conditions (22 ± 2°C, 12:12 light/dark cycle, ad libitum food and water) with 4 mice per cage. Mice were randomly divided into four groups (n=7 per group) using a computer-generated random number sequence (Microsoft Excel, RAND function). Group allocation was performed by an investigator not involved in the animal care or outcome assessment. Groups 1, 2, and 3 were subcutaneously injected with 2×10^6 attenuated *N. caninum* tachyzoites (strain Nc-1) combined with micronized chitosan adjuvant (molecular weight: [X] kDa, degree of deacetylation: [Y]%, particle size: [Z] µm, supplier: [Company]) at concentrations of 10%, 50%, and 90% (w/v of final formulation), respectively. Group 4 served as the negative control and did not receive any vaccine formulation or adjuvant. Each mouse

received a single 100 µL subcutaneous injection in the dorsal neck region. Animals were monitored daily for three weeks to assess their general health status (body weight, behavior, injection site reaction, and clinical signs). At the end of the experimental period (3 weeks post-immunization), blood samples were collected from all mice via cardiac puncture. Serum was separated and stored at -20°C until subsequent immunological analyses. The laboratory technician performing the ELISA and IFN-γ assays was blinded to the group assignments until all data were collected.

Antigen preparation and ELISA test

Neospora antigen was prepared by sonicating 2×10^9 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. For ELISA, microtiter plates were coated with 100 µL of antigen (10 µg/well) diluted in bicarbonate buffer, and incubated at 4°C for 24 hours. Plates were 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 using a microplate reader [6]. All samples were run in duplicate, and the mean absorbance was used. Blank wells (substrate only), negative serum control (from unimmunized mice), and positive serum control (from mice previously immunized with *N. caninum*) were included on each plate.

Gamma interferon (IFN-γ) assay

Serum IFN-γ levels were quantified using a commercial mouse IFN-γ ELISA kit (Invitrogen™ Mouse IFN-γ ELISA Kit, catalog # [X], Thermo Fisher Scientific, Waltham, MA, USA) based on monoclonal antibodies, following the manufacturer's protocol. The detection range was 15.6–1000 pg/mL with a sensitivity of <5 pg/mL. The required sample volume was determined according to the

Resource Equation Approach with E value = 24 (total animals 28, groups 4, so error df = 24). Samples were run in duplicate.

Data analysis

Statistical analysis was performedusing SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare group means, followed by Tukey's post-hoc test for multiple pairwise comparisons. Adjusted P values were calculated using Tukey's method. A p-value of less than 0.05 was considered statistically significant. Data are presented as mean ± SD unless otherwise stated. Each mouse was considered as one experimental unit.

Authors' Contributions

Seyed Reza Hosseini and Marzieh Kefayat planned the experiments. Milad Hamzehali Tehrani and Amirhosein Norouzi took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Competing Interests

The authors declare that there is no conflict of interest.

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