EVALUATION OF CHITOSAN NANOPARTICLES (CSNP) AS POTENT IMMUNE ADJUVANT FOR PESTE DES PETITS RUMINANTS (PPR) LIVE OCULAR VACCINE: A STEP FORWARD TOWARDS ERADICATION

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ABSTRACT

Peste des Petits Ruminants (PPR) is a highly contagious viral disease of small ruminants that causes significant economic losses and threatens global food security. Although the Nigeria 75/1 PPR vaccine is effective, improved immunization strategies are needed to induce mucosal immunity with an inherent potential to reduce virus shedding and transmission. This study evaluated the immune response induced by a Chitosan Nanoparticle (CSNP)-based PPR vaccine administered through intranasal (IN) and ocular (OC) routes in goats. Methods: CSNPs were synthesized using the ionic gelation method, confirmed sterile, and mixed with the Nigeria 75/1 PPR vaccine in equal proportions. Five groups were designed: G1 and G2 received the standard vaccine via IN and OC routes; G3 and G4 received the CSNP-based vaccine via the same routes; G5 served as the unvaccinated control. Immune response was assessed using white blood cell (WBC) counts, competitive ELISA (c-ELISA), and Virus Neutralization Test (VNT). Results: G3 and G4 demonstrated enhanced, persistent antibody responses and significantly higher WBC counts than other groups. At day 60 post-vaccination, c-ELISA values were highest in G3 (91.7) and G4 (91.6), while VNT results showed virus neutralization up to a 1:640 dilution for CSNP-based vaccines, compared with 1:80 for the standard vaccine. The control group remained negative throughout. These findings indicate that the CSNP-based PPR vaccine, particularly via the ocular route, enhances both humoral and mucosal immunity. This formulation represents a promising, sustainable approach for efficient vaccination and supports global efforts toward PPR eradication by 2030.

Keywords: PPR eradication, Mucosal vaccination, Immune adjuvant, Nanoparticle-based vaccine, CSNP-based PPR vaccine

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INTRODUCTION

The Peste des Petits Ruminants (PPR) is a highly contagious viral disease of small ruminants, including sheep and goats, and has caused significant economic repercussions for communities that largely depend on agricultural activities. Although vaccines are available, achieving long-lasting immunity and limiting the widespread spread of the disease remain challenges (Alamerew *et al*., 2025). Recent innovations in nanotechnology are useful in improving the efficacy of vaccines. Chitosan Nanoparticles (CSNP), which have gained attention and should be regarded as a promising option due to their biodegradable, biocompatible, and mucoadhesive characteristics, can be utilized as an effective immune adjuvant (Gong *et al*., 2022).

PPR is primarily associated with small ruminants (sheep and goats); however, cattle, pigs, and camels can also develop disease symptoms, affecting the livestock business (Tenuche *et al*., 2023). This highly contagious disease, also referred to as ovine rinderpest, goat plague, pneumoentritis syndrome, and infectious pustular stomatitis, has a great impact on the economic well-being of small ruminants (Kumar *et al*., 2014). Peste des Petits Ruminants Virus (PPRV) is a single-stranded RNA virus that is classified under the genus Morbillivirus of family Paramyxoviridae and is the cause of this viral disease. PPRV is a negative-sense, enveloped virus, and the structure of PPRV is characterized by several parts. For example, it has 14,948 non-segmented nucleotides in its genome, which is a negative-sense RNA. The genome is composed of six structural proteins and two non-structural proteins (Courcelle *et al*., 2024).

PPR is widely spread all over Southern Asia, the Middle East, and Africa (Abbas *et al*., 2012). PPR has clinical manifestations of high mortality rates, fever, vomiting, eye and nose discharge, mouth ulcers, pneumonia, and

eviscerating diarrhea. A high rate of abortions has also been proven to be caused by PPR infection in goats (Albina *et al*., 2013). Animals can be exposed to the PPRV virus through their nasal and oral tract. Upon entry into the body, the virus initially replicates in the respiratory and nasopharyngeal epithelium, then infects the lymphoid organs, where a second cycle of reproduction is observed (Albina *et al*., 2013).

Morbilliviruses primarily target lymphoid organs, and the immune system is largely weakened by the loss of white blood cells from infection. PPRV causes significant clinical symptoms during the acute stage, where the specific symptom varies depending on the species, age, virulence of the strain, and the presence of other infectious agents (Abubakar *et al*., 2012). This disease is characterized by fever, breathing difficulties, anorexia, deeper depression, erosive stomatitis, eye and nose inflammation, and excessive watery diarrhea, which can be contaminated with blood. In the more severe cases, bacterial secondary infections that are related to immunosuppression are likely to cause severe bronchopneumonia. The PPRV is detected in several body fluids, and viral shedding of infected animals usually takes place between 3 and 22 days after infection (Abubakar & Munir, 2014).

The traditional live attenuated injectable vaccines have been the backbone of the PPR control. The most commonly used strain of vaccine is the PPRV Nigeria 75/1 vaccine, which is effective in most areas, resulting in a significant reduction in the disease rate. Nevertheless, these conventional vaccines have several limitations such as thermolability, logistics, stability, and the required cold chain facility that affect the efficacy of the vaccines and restrict their broader implementation (Fanelli *et al*., 2022). To achieve the aim of global PPR eradication, mass vaccination of small ruminant populations through simple administration routes, i.e., intra-ocular or intra-nasal delivery, will facilitate vaccine deployment. These approaches require minimal equipment, are easy to carry out in field settings, and induce mucosal immunity, which is essential for protection against PPR infection (Kabir *et al*., 2025; Zahid & Latif, 2025).

Mucosal immunity is the immunological response of the mucous membranes of the respiratory, gastrointestinal, and urogenital tracts. This immunity is essential to prevent the entry of pathogens into the initial site, thereby preventing their spread throughout the body. The virus typically enters the organism through the oral, nasal, or ocular mucous membranes and spreads to the lymphatic system, lungs, and gastrointestinal tract (Chukwudi *et al*., 2025). Therefore, the vaccines resulting in the development of strong mucosal immunity excreted at these locations would be more effective in the prevention of the clinical disease, as well as virus spread (Turkar & Devi, 2024).

Nanoparticle-based vaccines (NBVs), a new class of vaccine therapeutic delivery technologies that use nanoparticles as vectors to carry antigens and immune-activating components. These nanoparticles may consist of different materials (lipids, polymers, proteins, or inorganic compounds such as gold, silica, or iron oxide). The peculiarity of NBVs is that they can react with immune cells through new mechanisms and penetrate bio-barriers, where larger molecules cannot penetrate (Bezbaruah *et al*., 2022). NBVs have several benefits in comparison with traditional methods, including live attenuated or inactivated vaccines. These advantages are of utmost significance due to the challenges posed by diseases like PPR, control of which requires safe, effective, and stable vaccines (Bezbaruah *et al*., 2022).

Chitosan is a natural biopolymer derived from Chitin and has emerged as a promising material in the development of vaccines. Its appeal is also associated with a number of major characteristics, such as, excellent biocompatibility, biodegradability, low toxicity, and easy modification to numerous pharmaceutical purposes (Kole *et al*., 2019). CSNP has emerged as a promising vaccine delivery system since it can optimize both humoral and cellular immunity. CSNP can promote local immunity and is adapted to mucosal surfaces, such as the respiratory and gastrointestinal mucous membranes, which are the main entry points for several pathogens (Mura *et al*., 2022). The CSNP are designed to release antigens slowly so they may act on the immune system over a long period, thus resulting in better and sustained immunity (Malek-Khatabi *et al*., 2022). Figure 1 illustrates a brief schematic outline of the study, highlighting the development and immunological evaluation of chitosan nanoparticles (CSNP) as an adjuvant for the PPR vaccine.

CSNP-based vaccines elicit antibodies by improving the immunogenicity and delivery of viral antigens to the immune system. Viral antigens are encapsulated or adsorbed to CSNP, which serves as an antigen carrier and adjuvant in promoting the uptake of the antigens by antigen-presenting cells (APCs), including dendritic cells and macrophages (Chattopadhyay *et al*., 2017). After endocytosis, the CSNPs degrade the viral antigens in the endosomal compartments and are processed into peptide fragments and loaded onto major histocompatibility complex class (MHC) molecules. The antigen-MHC complexes are then displayed on the APC surface and recognized by $CD4^+$ helper T cells, which are subsequently activated (Wen *et al*., 2011). Activated helper T cells deliver crucial co-stimulatory signals and cytokines to B lymphocytes, which identify the same antigen, facilitate B-cell activation, clonal expansion, and B-cell differentiation to plasma cells (Salamanca *et al*., 2006). The plasma cells then release antibodies specific to the virus, which lead to successful humoral immunity and neutralize any virus particle, and lead to protective immunity (de Salamanca *et al*., 2006; Gong *et al*., 2022; Wen *et al*., 2011).

This study aims to evaluate the potential of CSNP as an immune adjuvant in enhancing the immunogenicity and protection of PPR vaccines, thereby bolstering disease management strategies in endemic regions. In another baseline study at the Transboundary Animal Disease (TAD) Lab, Department of Microbiology, and University Diagnostic Lab, CUVAS, we conducted lab animal trials of PPRV live vaccine through subcutaneous, ocular, and nasal routes and later conducted field trials at a private farm (data not presented here). In our field trials, we observed that the subcutaneous vaccine resulted in seroconversion for a longer period compared to the ocular and nasal administration routes. However, it is quite apparent that the ocular route has the capacity to induce a mucosal immune response. To address the shortfall in immune efficacy seen with the ocular vaccine, we developed a pre-clinical trial leveraging a CSNP-based vaccine administered via OC and IN routes.

MATERIALS AND METHODS

Chitosan Nanoparticles (CSNP) Preparation: Low molecular weight Chitosan (white to light tan powder form; low molecular weight of 50,000-190,000 Da) sourced from the local market (CAS# 9012-76-4) was used for the preparation of Chitosan Nanoparticles (CSNP) by Ionic gelation, following the protocol described by (Calvo *et al*., 1997) (Figure 2). In brief, 25mg chitosan powder was dissolved in 1% acetic acid solution to obtain a 0.05% (w/v) solution, stirred for 24 h, and the pH was adjusted to 5.5 ± 0.1 using 1 M NaOH under continuous magnetic stirring. A 0.1% (w/v) sodium tripolyphosphate (STPP) solution was then added dropwise at a rate of 0.3ml/minute, and the resulting mixture was centrifuged at 15,000 rpm for 30 minutes at room temperature (25 ± 2°C). The nanoparticle pellet was washed with deionized water to remove any unbound STPP. The CSNP suspension was sterilized using a 0.22-micron filter (Van Buynder *et al*., 2013), and stored at 4°C until further use.

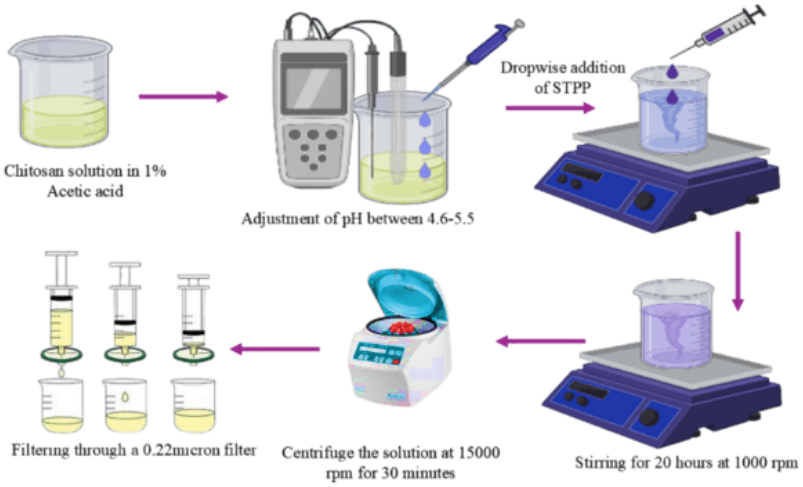

Figure 2. Preparation of Chitosan Nanoparticles (CSNP) by the Ionic gelation method

Sterility Test: The CSNP preparation was tested for sterility (aerobic and anaerobic bacteria and fungi) by inoculating 100 µL of CSNP into Tryptic Soy Broth (TSB) and Fluid Thioglycolate Medium (FTM), then incubating at 37°C for 48 hours. The culture tubes were observed for 3-4 days for TSB and for 14 days for FTM. The Sterility of the CSNP was confirmed by the absence of microbial growth (Rweyemamu *et al*., 1994).

CSNP Characterization: The characterization of CSNP was not performed in the present study due to limited access to instrumentation such as dynamic light scattering (DLS) and zeta sizer systems. Importantly, our synthesis strictly followed well-established protocols reported in the literature, including classical procedures originally described by Calvo *et al*. and extensively adopted in subsequent studies, shown to yield stable CSNP with mean sizes around ~200 nm with PDI values below ~0.3 and zeta potentials in the positive range (often > +30 mV) (Calvo *et al*., 1997; Hoang *et al*., 2022; Kumar *et al*., 2014; Van Bavel *et al*., 2023). This limitation reflects restrictions in local analytical resources rather than a lack of recognition of their importance (Agarwal *et al*., 2018; Calvo *et al*., 1997).

CSNP-based PPR Vaccine Formulations: The lyophilized Nigeria 75/1 PPR vaccine (10^5 $TCID_{50}$/mL), manufactured by Nuclear Institute for Agriculture and Biology, Faisalabad (NIAB), which is registered and routinely used for intra-ocular administration in small ruminants, was used for experimental evaluation. For reconstitution of the freeze-dried vaccine, 500 µL of the provided diluent was added, along with 500 µL of CSNP solution, to obtain a 1:1 ratio. The reconstituted experimental vaccine formulation thus contained a standard dose of 10^4 $TCID_{50}$/100 µl of PPRV and

2.5mg/100 µl of CSNP. 100 µl of this vaccine per animal was administered via intraocular and intranasal routes. The antigen was associated with the nanoparticles through physical adsorption mediated by electrostatic interactions, without chemical conjugation or encapsulation (Gong *et al*., 2022). This ratio was selected to ensure uniform antigen distribution while preserving vaccine potency and nanoparticle stability. Mixing was carried out aseptically at room temperature with gentle agitation prior to administration. Intranasal administration was performed using a low-volume drop-wise method, avoiding aerosolization, while intra-ocular administration followed the manufacturer's recommended protocol (Dmour & Islam, 2022; Janovska *et al*., 2023; Renu & Renukaradhya, 2020).

Experimental Animals, Grouping, and Vaccinations: A total of 15 goats of the same breed, apparently healthy, with age of 6-7 months, were bought from the local market. All animals were screened and confirmed to be free from PPR infection using competitive ELISA (c-ELISA) prior to inclusion. For at least a week before the experiment began, all animals were housed and managed in the same way to help them become accustomed to the new husbandry routine, in accordance with ethical considerations (Approval #ORIC-68). A brief summary of the experimental animals' information is presented in Table 1. All animals were determined to be healthy and disease-free over that period, with rectal temperatures on average between 38.4°C and 39.6°C. Using c-ELISA, serum samples from every animal were collected and examined for the presence of antibodies against the PPR virus. All of the animals had percentage inhibition (PI) values well below 50% (average of 21%), indicating that they were negative for PPR. Seven days before immunization, all of the animals were dewormed using ITRIC oral suspension, which included triclabendazole and ivermectin (5 milliliters per animal). The fifteen goats were distributed into five groups, with three animals in each group. The treatment groups, including the route and dosage of the vaccine, are summarized in Table 2. The vaccine was administered via the intranasal (l ml/animal as a 100 µl vaccine having 10^4 $TCID_{50}$/100 µl was diluted to 1ml) and intra-ocular routes (50 µl/eye having 10^4 $TCID_{50}$/100 µl) using a sterile needle-free delivery system. For intranasal administration, the vaccine was delivered dropwise into the nostrils, while intra-ocular administration was performed by applying it dropwise to the conjunctival sac, following standard mucosal vaccination procedures. The difference in administration volume between the intranasal (1 mL/animal in diluted form having the same concentration of virus used per eye) and ocular (50 µL/eye) routes was intentional and based on established physiological and practical constraints, as conjunctival vaccination in small ruminants is routinely performed using ~50 µL per eye to avoid overflow and ensure mucosal retention (Barisani-Asenbauer *et al*., 2013; Gurbilek *et al*., 2023). There were no changes in the goats' body temperature or any clinical symptoms over the 48 hours after inoculation.

Table 1. Experimental Animals Information

Parameter	**Details**
Total number of goats	15
Breed	Same breed (Makhi Cheeni)
Age	6–9 months
Health status confirmed by c-ELISA	Healthy, disease-free (pI≤50)
Acclimatization period	1 week
Rectal temperature range	38.4°C – 39.6°C
Antibody test for PPRV	All negative (PI ≤ 50 via c-ELISA)
Deworming (standard prophylactic management)	5 ml per animal ITRIC oral suspension (Triclabendazole + Ivermectin)
Deworming timing	7 days before vaccination

Table 2. Treatment groups with route and dosage of vaccine

Group#	**No. of Animals**	**Vaccine Type**	**Route**	**Dosage**	**Vaccine Concentration**/100 µl	**Notes**
1	3	Nigeria 75/1 PPR	Intranasal (IN)	100 µl /animal (Diluted to 1ml volume)	10^4 $TCID_{50}$	Standard vaccine
2	3	Nigeria 75/1 PPR	Ocular (OC)	50 µl/eye	10^4 $TCID_{50}$	Standard vaccine
3	3	CSNP-based Nigeria 75/1 PPR	Intranasal (IN)	100 µl /animal (Diluted to 1ml volume)	$10^4$$TCID_{50}$	CSNP-based vaccine
4	3	CSNP-based Nigeria 75/1 PPR	Ocular (OC)	50 µl/eye	10^4 $TCID_{50}$	CSNP-based vaccine
5	3	None (Control)	—	—	—	No vaccine

Sample Collection and Transportation: Blood samples of each vaccinated animal were collected before vaccination and post-vaccination from the jugular vein (Figure 3) at intervals (0, 2, 7, 14, 21, 28, 38 and 60 days) (Stayt, 2022), and transported in a cold chain to the Department of Microbiology at Cholistan University of Veterinary and Animal Sciences, Bahawalpur, and serum was harvested and stored at -20°C for further analysis in serological and neutralization tests. The limited sample size was selected for this preliminary exploratory immunogenicity study in accordance with ethical and logistical constraints.

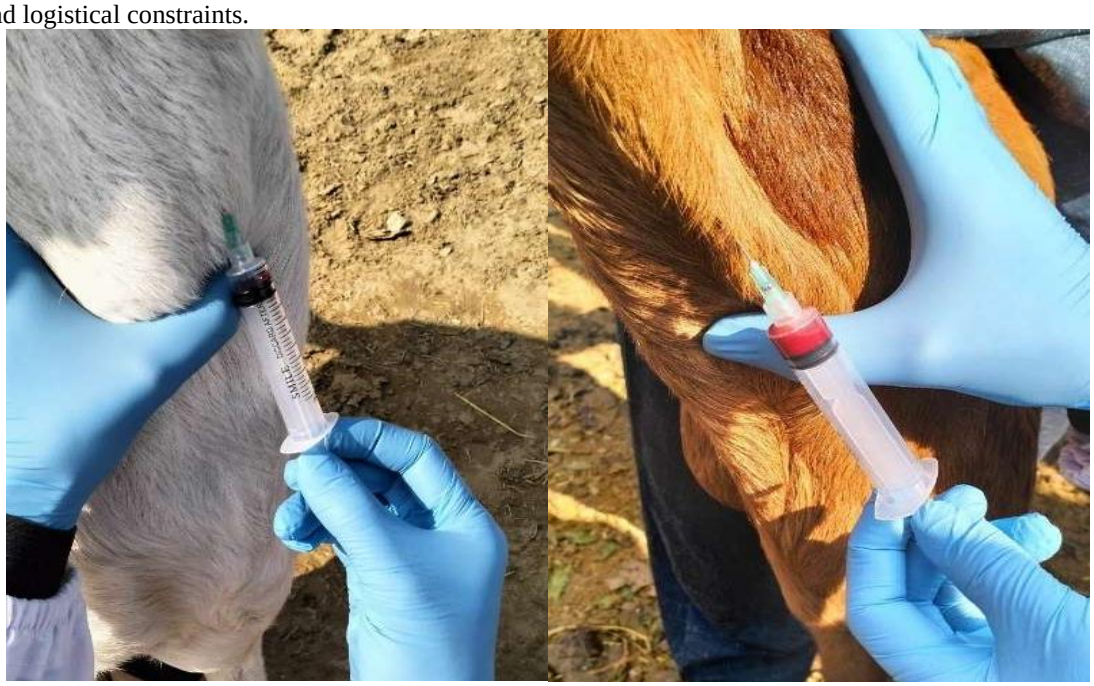

Figure 3. Collection of blood from jugular vein for hematology and serum isolation

Hematology (White Blood Cell (WBC) Count): The blood samples at 0, 2, 7, 14, 21, 28, 38, and 60 days post-vaccination were collected for hematological analysis. The WBC was measured by the manual method using a hemocytometer, as automated hematology analyzers were not available for goat species, following the method described by Lutz & Dzik (Lutz & Dzik, 1993). We diluted the blood to 1:20 with normal saline, 1% acetic acid to lyse red blood cells, and 0.2µl of Giemsa stain to stain the white blood cells. A small drop (10µL) of diluted blood was placed on the counting chamber of the hemocytometer and observed under the microscope. WBCs were counted, and the total was calculated to estimate the WBC count in the blood. A standard hemocytometer has one corner grid of 16 squares (1mm x 1mm in area and 0.10 mm deep), and the dilution factor is 1:20. The calculation formula for WBC count is:
WBC count = Number of WBCs counted × Dilution Factor × 10^4 / Volume of blood

Competitive Enzyme-Linked Immunosorbent Assay (c-ELISA): Serum samples were analyzed for PPR-specific antibodies using a commercial competitive ELISA kit (ID Screen® PPR Competition, IDvet, Grabels, France), following the manufacturer's instructions. Positive and negative control sera provided with the kit were included in each run for validation. Results were expressed as percent inhibition (PI), with samples showing PI ≤ 50% considered negative and PI > 50% considered positive for PPR antibodies.

Virus Neutralization Test (VNT): Virus neutralization tests (VNT) were conducted using the Nigeria 75/1 PPR virus strain. Serum samples were heat-inactivated at 56 °C for 30 min and subjected to serial two-fold dilutions before incubation with a fixed virus dose. The serum–virus mixtures were subsequently added to susceptible cell cultures maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum. Virus control, cell control, and positive control sera were included in each assay. Neutralizing antibody titers were determined based on the absence of cytopathic effects. The VNT was performed at the National Institute for Biotechnology and Genetic Engineering (NIAB), Pakistan, in accordance with the standard protocol described by the European Union Reference Laboratory for Peste des Petits Ruminants (EURL-PPR) (Raj *et al*., 2000).

Statistical Analysis: IBM SPSS Statistics (version 27.1) was used to analyze the data. The data were summarized using descriptive statistics, and the results are expressed as a mean and standard deviation. The Shapiro-Wilk test was used to

evaluate the data normality. The analysis of differences between groups was performed with one-way analysis of variance (ANOVA), and the test of the honestly significant difference (HSD) was used to perform a post hoc multiple comparison with the required results in several situations. Repeated-measures ANOVA was used to analyze repeated-measures data that were collected through weekly sampling. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

General Observations and Safety: No experimental goats showed adverse reactions throughout the study period, and all of them were clinically healthy after vaccination by intraocular or intranasal routes. There were no signs of local irritation, nasal clearance, ocular inflammation, or systemic illness, indicating that vaccine preparations were well tolerated.

Sterility Test of CSNP: After a 48-hour incubation period, no bacterial or fungal growth was observed in the CSNP solution, demonstrating that CSNP is sterile and can be used in vaccine formulation. The vaccine was reconstituted, after which the CSNP and vaccine were combined in equal proportions with a standard concentration.

Immunization and Clinical Observations: In all the experimental animals, PPR live vaccine (Nig. 75/1 strain) was administered through the ocular and IN routes (Figure 4). No negative clinical symptoms of vaccination, including fever and abnormal behavior, were observed. All the vaccinated groups (Groups 1 to 4) were observed for 48 hours after the vaccination and did not demonstrate any significant changes in body temperature, which indicated that the vaccine formulations were safe. All the animals were also disease-free and healthy, with an average rectal temperature of 38.4°C to 39.6°C during the study period.

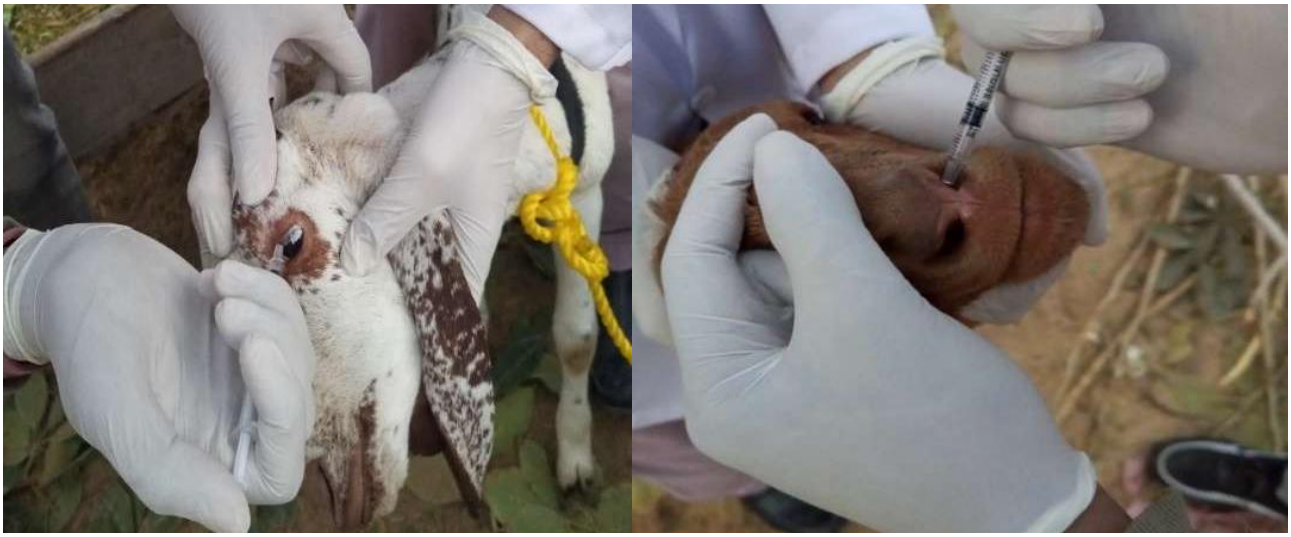

Figure 4. Administration of the vaccine through the Ocular and IN route

Hematology (WBC Count): The baseline values of WBC were within the normal range of physiological values in all animals before the vaccination (day 0), with no significant differences across groups ($p > 0.05$). After vaccination, there was a transient rise in WBC counts from day 0 to day 60 in all of the vaccinated groups, which is indicative of an immune response (Figure 5).

The statistical analysis showed that there was a significant difference in WBC counts, depending on the vaccination and time (repeated-measures ANOVA, $p \leq 0.05$). Animals that received the CSNP-adjuvanted PPR vaccine (Groups 3 and 4) showed a much greater and more persistent increase in the number of WBC than animals vaccinated with the non-adjuvanted vaccine (Tukey HSD, $p \leq 0.05$). It was found that peak levels of WBC were recorded on day 7 following vaccination in all vaccinated groups, but Groups 3 and 4 maintained high counts of WBC as late as day 14, while other groups showed a decreasing trend towards baseline levels.

At 28 days post-vaccination, WBC counts were back to baseline in most groups, although Groups 3 and 4 showed a slightly significant elevation. This prolonged leukocytic reaction indicates an augmented systemic immune stimulation concerning CSNP-based vaccine preparations, presumably due to improved antigen presentation and immune stimulation.

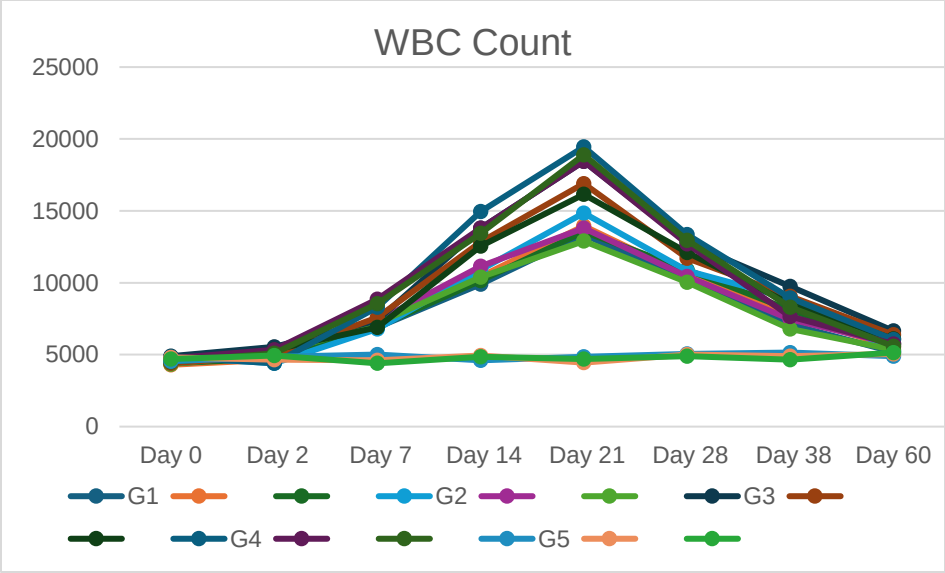

Figure 5. White Blood Cell counts from day 0 to day 60

Table 3. Descriptive Statistics of WBC count for each day of measurement

Day	Mean	Std Dev	Min	Max
Day 0	4620.0	198.03	4300.0	4900.0
Day 2	4960.0	350.1	4400.0	5550.0
Day 7	6926.67	1328.52	4400.0	8850.0
Day 14	10583.33	3364.93	4600.0	14950.0
Day 21	13646.67	5130.39	4450.0	19450.0
Day 28	10233.33	2916.44	4900.0	13350.0
Day 38	7533.33	1575.1	4650.0	9750.0
Day 60	5636.67	507.26	4900.0	6650.0

Std Dev = Standard Deviation, Min = Minimum, Max =Maximum

Table 3 summarized the descriptive statistics of the average standard deviations, minimum, and maximum values for each day. The mean values for Days 0, 2, 7, 14, 21, 28, 38, and 60 show a trend of increasing values over time, especially in the initial period (Day 0 to Day 7), with a peak around Day 21.

Table 4. Results of the Shapiro-Wilk Normality Test

Day	Test Statistic	p-value
Day 0	0.938	0.354
Day 2	0.957	0.648
Day 7	0.89	0.067
Day 14	0.871	0.034
Day 21	0.841	0.013
Day 28	0.806	0.004
Day 38	0.913	0.15
Day 60	0.963	0.746

As shown in Table 4, the Shapiro-Wilk test was used to assess the normality of the data distributions for Days 0, 2, 7, 38, and 60, which follow a normal distribution (p-value > 0.05), while the data for Days 14, 21, and 28 do not follow a normal distribution (p-value ≤ 0.05).

Table 5. Results of the One-Way ANOVA Test

Day	F-statistic	p-value
Day 0	0.484	0.748
Day 2	0.86	0.52
Day 7	76.528	≤0.001
Day 14	150.412	≤0.001
Day 21	167.26	≤0.001
Day 28	208.134	≤0.001
Day 38	16.732	≤0.001
Day 60	12.264	0.001

One-way ANOVA tests in Table 5 reveal that there is no significant difference between the groups at Days 0 and 2 (p-values > 0.05). However, significant differences were found at Days 7, 14, 21, 28, 38, and 60 (p-values ≤ 0.05), indicating that the groups' measurements significantly changed over time, particularly after Day 7.

Table 6. Multiple Comparison of Means at day 7- Tukey HSD, FWER=0.05

Comparison	Mean Difference	Adjusted p-value	Lower CI	Upper CI	Significant (Reject H0)
G1 vs G2	133.33	0.9795	-664.56	931.23	No
G1 vs G3	316.67	0.6939	-481.23	1114.56	No
G1 vs G4	1633.33	0.0004	835.44	2431.23	Yes
G1 vs G5	-2316.67	0.0000	-3114.56	-1518.77	Yes
G2 vs G3	183.33	0.9375	-614.56	981.23	No
G2 vs G4	1500.00	0.0008	702.11	2297.89	Yes
G2 vs G5	-2450.00	0.0000	-3247.89	-1652.11	Yes
G3 vs G4	1316.67	0.0021	518.77	2114.56	Yes
G3 vs G5	-2633.33	0.0000	-3431.23	-1835.44	Yes

Lower CI, =Lower Confidence Interval, Upper CI, =Upper Confidence Interval

On Day 7, significant differences were found between the treatment groups and the control, while group G4 showing the significant response. Vaccinated groups (G1-G3) had similar outcomes but all performed significantly better than the unvaccinated control as shown in Table 6.

Table 7. Multiple Comparison of Means at day 21- Tukey HSD, FWER=0.05

Comparison	Mean Difference	Adjusted p-value	Lower CI	Upper CI	Significant (Reject H0)
G1 vs G2	233.33	0.9944	-1746.19	2212.85	No
G1 vs G3	3550.00	0.0011	1570.48	5529.52	Yes
G1 vs G4	5316.67	0.0000	3337.15	7296.19	Yes
G1 vs G5	-8950.00	0.0000	-10929.52	-6970.48	Yes
G2 vs G3	3316.67	0.0019	1337.15	5296.19	Yes
G2 vs G4	5083.33	0.0001	3103.81	7062.85	Yes
G2 vs G5	-9183.33	0.0000	-11162.85	-7203.81	Yes
G3 vs G4	1766.67	0.0864	-212.85	3746.19	No
G3 vs G5	-12500.00	0.0000	-14479.52	-10520.48	Yes
G4 vs G5	-14266.67	0.0000	-16246.19	-12287.15	Yes

Lower CI, =Lower Confidence Interval, Upper CI, =Upper Confidence Interval

On Day 21, the Tukey HSD test showed no significant difference between groups G1 and G2 (p = 0.9944) and between groups G3 and G4 (p = 0.0864), indicating similar outcomes within these pairs. Significant differences were observed when comparing G1 and G2 to G3 and G4, showing notably higher mean values (p ≤ 0.01).

Table 8. Multiple Comparison of Means at day 60- Tukey HSD, FWER=0.05

Comparison	Mean Difference	Adjusted p-value	Lower CI	Upper CI	Significant (Reject H0)
G1 vs G2	-66.67	0.9963	-696.86	563.53	No
G1 vs G3	866.67	0.0076	236.47	1496.86	Yes
G1 vs G4	233.33	0.7418	-396.86	863.53	No
G1 vs G5	-483.33	0.1607	-1113.53	146.86	No
G2 vs G3	933.33	0.0045	303.14	1563.53	Yes
G2 vs G4	300.00	0.5473	-330.19	930.19	No
G2 vs G5	-416.67	0.2629	-1046.86	213.53	No
G3 vs G4	-633.33	0.0487	-1263.53	-3.14	Yes
G3 vs G5	-1350.00	0.0003	-1980.19	-719.81	Yes
G4 vs G5	-716.67	0.0248	-1346.86	-86.47	Yes

Lower CI, =Lower Confidence Interval, Upper CI, =Upper Confidence Interval

Groups G1 and G2 had no significant difference (p=0.9963) on Day 60, and this meant that the results were similar. But G3 had higher mean values than G1 and G2 (p=0.0076 and 0.0045, respectively), indicating a greater effect in G3. G3 and G4 had considerably greater values as compared to the control group (G5) (p=0.0003 and 0.0248), which proves the effectiveness of treatments in the groups. Overall, it reveals that G3 is the most effective treatment option, and it is followed by G4, significantly differentiating control and vaccinated groups as presented in Table 8.

c-ELISA: The c-ELISA results demonstrate the antibody response kinetics of the experimental groups within the day 0-60 study period. Seropositivity was defined as a cut-off value of ≥50% inhibition (%PI). The PI values for all vaccinated groups (G1, G2, G3, G4) were well below the cut-off value (Average PI=21%) on day 0, confirming the absence of Anti-PPRV antibodies. By day 7, a significant rise in antibody levels (PI=56.10%, P value ≤0.001) was noted, especially in ocular (G2) and CSNP- adjuvated groups (G3 and G4). Peak responses (PI=72.72% and 72.96%, P value ≤0.001) were observed between days 14-28. The CSNP-adjuvanted groups retained higher percentages of PI>72% than non-adjuvanted ones as indicated in line charts of c-ELISA of vaccine trial in goats via ocular and IN routes (Figure 6).

Since the 14th day, the PI values of all the vaccinated groups were high, which indicates the continued antibody response during the period of observation. The repeated-measures analysis indicated a significant effect of the vaccination across time (p≤0.05), whereas goats vaccinated with the CSNP-based PPR formulations (G3 and G4) had higher Peak PI values of (Average PI=91.65%) with an approximate 1 log difference compared to those in the non-adjuvanted vaccine groups (Average PI=80.45%) (Figure 6).

From day 21 to day 60, the antibody levels of all vaccinated groups were found to be stable, which shows long-term serological immunity. A temporary reduction in antibody levels was observed around day 38 in vaccinated groups, but they returned by day 60, particularly in G3 (IN + CSNP) and G4 (OC + CSNP), which had the highest percentage of inhibition. Such fluctuations are not uncommon in longitudinal serological studies and may reflect biological variability, assay sensitivity, or redistribution of circulating antibodies. Although the control group (G5) was seronegative during the period of the study.

Table 9. Results of the Shapiro–Wilk Normality Test for c-ELISA PI Values

Day	Test Statistic (W)	p-value	Interpretation
Day 0	0.94	0.56	Normal
Day 7	0.92	0.48	Normal
Day 14	0.96	0.67	Normal
Day 21	0.97	0.71	Normal
Day 28	0.95	0.62	Normal
Day 38	0.93	0.54	Normal
Day 60	0.96	0.69	Normal

The Shapiro-Wilk test of normality revealed that percent inhibition (PI) values on all days of sampling (Day 0 to Day 60) were normally distributed, with the p-values being more than 0.05 at every time point. This indicates that the data were in accordance with the assumptions to apply the parametric statistical tests, such as one-way ANOVA and HSD post-hoc test.

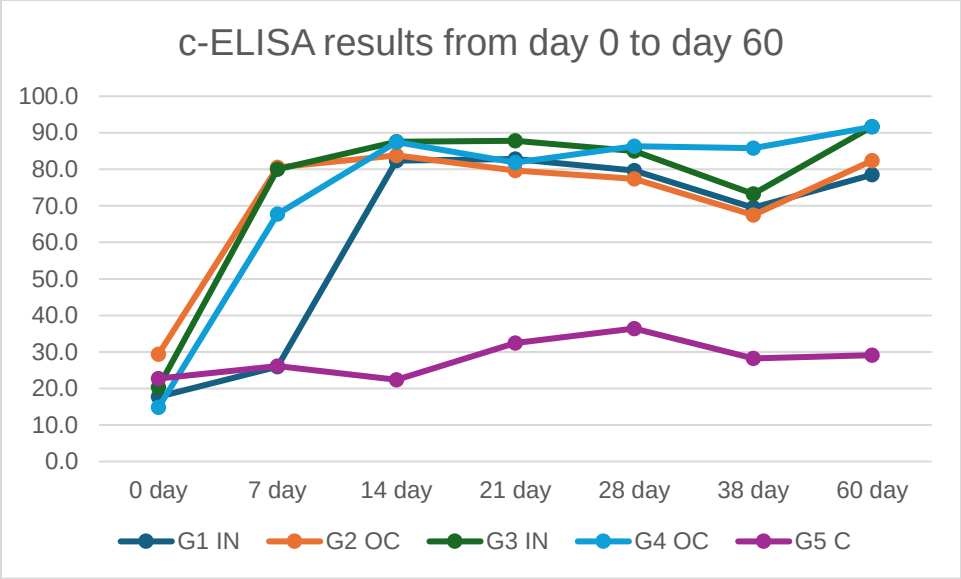

Figure 6: Line charts of c-ELISA of vaccine trial in goats via ocular and IN routes

Table 10. Summary of statistical analysis of c-ELISA results across different days

Day	Mean	Std Dev	Min	Max
0 day	21.00	5.55	14.80	29.40
7 days	56.10	27.85	26.00	80.50
14 days	72.72	28.22	22.40	87.50
21 days	72.92	22.84	32.40	87.80
28 days	72.96	20.76	36.40	86.30
38 days	64.86	21.69	28.20	85.80
60 days	74.66	26.11	29.10	91.70

The mean values were much higher on Day 14 (mean = 72.72) compared to Day 0 (mean = 21.00) and did not significantly vary on Days 21 and 28, which proves the presence of a strong immune response that develops after vaccination. There was a minor decrease on Day 38 (mean = 64.86) and a resurgence on Day 60 (mean = 74.664), indicating enhanced immune activity. Standard deviation values were greater at intermediate periods of time (e.g., 27.85 on Day 7 and 28.22 on Day 14), which shows variability in the response of animals, but decreased slightly towards the later days.

Table 11. One-Way ANOVA of c-ELISA Percent Inhibition (%PI) Values Across Time

Day	df (Between, Within)	F value	p-value	Significance
0	(4, 10)	1.82	0.21	NS
7	(4, 10)	96.4	≤ 0.001	***
14	(4, 10)	182.7	≤ 0.001	***
21	(4, 10)	222.7	≤ 0.001	***
28	(4, 10)	165.3	≤ 0.001	***
38	(4, 10)	141.9	≤ 0.001	***
60	(4, 10)	198.6	≤ 0.001	***

NS = Not significant $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

One-way ANOVA revealed no significant differences between experimental groups at Day 0, which proved the homogeneity of the baseline and the absence of pre-existing antibodies before vaccination, as shown in Table 11. Since Day 7, there were highly significant differences ($p \leq 0.001$) between groups in all subsequent time points (Days 7, 14, 21, 28, 38, and 60). This reflects that the vaccination groups (G3 and G4) had a significantly higher level of antibody production, and divergence increased over time between vaccinated and control groups.

Table 12. Comprehensive Tukey's HSD Post-Hoc Comparison of c-ELISA PI Values Across Time

Day	G1 vs G2	G1 vs G3	G1 vs G4	G1 vs G5	G2 vs G3	G2 vs G4	G2 vs G5	G3 vs G4	G3 vs G5	G4 vs G5
0	0.62 (NS)	0.71 (NS)	0.54 (NS)	0.66 (NS)	0.59 (NS)	0.48 (NS)	0.61 (NS)	0.57 (NS)	0.64 (NS)	0.52 (NS)
7	≤0.001***	≤0.001***	0.002**	0.78 (NS)	0.91 (NS)	0.004**	≤0.001***	0.006**	≤0.001***	≤0.001***
14	0.67 (NS)	0.003**	0.004**	≤0.001***	0.09 (NS)	0.11 (NS)	≤0.001***	0.98 (NS)	≤0.001***	≤0.001***
21	0.41 (NS)	0.004**	0.52 (NS)	≤0.001***	≤0.001***	0.97 (NS)	≤0.001***	0.002**	≤0.001***	≤0.001***
28	0.58 (NS)	0.006**	0.004**	≤0.001***	0.003**	0.002**	≤0.001***	0.87 (NS)	≤0.001***	≤0.001***
38	0.64 (NS)	0.002**	≤0.001***	≤0.001***	0.001**	≤0.001***	≤0.001***	0.94 (NS)	≤0.001***	≤0.001***
60	0.59 (NS)	≤0.001***	≤0.001***	≤0.001***	≤0.001***	≤0.001***	≤0.001***	0.96 (NS)	≤0.001***	≤0.001***

G1 = IN | G2 = Ocular | G3 = IN + CSNP | G4 = Ocular + CSNP | G5 = Control
NS = Not significant, $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

Tukey's HSD multiple comparison test revealed no significant differences among the experimental groups at day 0, confirming comparable baseline antibody levels shown in Table 12. From day 7 to day 60, significant group-wise differences were observed, with all vaccinated groups (G1–G4) showing significantly higher PI values than the control group (G5). The CSNP-adjuvanted groups (G3 and G4) consistently exhibited significantly higher antibody responses than the non-adjuvanted groups (G1 and G2). Differences between the intranasal (G1) and ocular (G2) routes without adjuvant were largely non-significant as compared to CSNP adjuvanted groups. Comparisons between the intranasal and ocular CSNP-adjuvanted groups (G3 vs. G4) were also mostly non-significant, indicating comparable efficacy of both routes when combined with CSNP. Overall, the CSNP adjuvant was the primary factor influencing the magnitude and persistence of the antibody response.

Virus Neutralization Test (VNT): At day 0, all groups (G1–G5) showed cytopathic effects at all tested serum dilutions (1:10–1:640), confirming the absence of pre-existing neutralizing antibodies against PPR virus (Table 13). By day 60 post-vaccination, clear differences in neutralizing antibody responses were observed among groups. Goats vaccinated with standard PPR vaccine (G1 and G2) neutralized the virus at a maximum serum dilution of 1:80 and 1:160, respectively, which showed that moderate neutralizing immunity had been induced. Conversely, goats vaccinated with the CSNP-adjuvanted PPR vaccine (G3 and G4) demonstrated total neutralization at all dilutions to 1.640 in the neutralization test, indicating a significant functional antibody response.

The control group (G5), which was not vaccinated, exhibited cytopathic effects across all dilutions, confirming that no immunity was induced. The comparative analysis of the results of VNT and c-ELISA (Table 14) shows that both formulations led to the development of humoral responses. While the production of neutralizing antibodies after CSNP-based vaccination was higher, indicating enhanced functional immunity and a potential for improved protective efficacy.

Table 13. VNT results of all groups at 0 and 60 days post-vaccination with standard and CSNP-based PPR vaccine

Groups-Day	1:10	1:20	1:40	1:80	1:160	1:320	1:640	Control
1-0	+	+	+	+	+	+	+	+
2-0	+	+	+	+	+	+	+	+
3-0	+	+	+	+	+	+	+	+
4-0	+	+	+	+	+	+	+	+
5-0	+	+	+	+	+	+	+	+
1-60	-	-	-	-	+	+	+	+
2-60	-	-	-	-	-	+	+	+
3-60	-	-	-	-	-	-	-	+
4-60	-	-	-	-	-	-	-	+
5-60	+	+	+	+	+	+	+	+

"+" denotes cytopathic effects, "-'' denotes virus neutralization

Table 14. Comparison table of VNT and c-ELISA results of all groups at 0 and 60 days post-vaccination with standard and CSNP-based PPR vaccine

Groups-Day	Vaccine Type	Time Point	c-ELISA PI (%)	c-ELISA Result	VNT Titer	VNT Result
1-0	Standard PPR IN	0 Day	17.7	-	≤1:10	-
2-0	Standard PPR OC	0 Day	29.4	-	≤1:10	-
3-0	CSNP-based PPR IN	0 Day	20.4	-	≤1:10	-
4-0	CSNP-based PPR OC	0 Day	14.8	-	≤1:10	-
5-0	Control No Vaccine	0 Day	22.7	-	≤1:10	-
1-60	Standard PPR IN	60 Day	78.5	+	1:80	∗+
2-60	Standard PPR OC	60 Day	82.4	++	1:160	++
3-60	CSNP-based PPR IN	60 Day	91.7	+++	≥1:640	+++
4-60	CSNP-based PPR OC	60 Day	91.6	+++	≥1:640	+++
5-60	Control No Vaccine	60 Day	29.1	-	≤1:10	-
∗+ indicate mild positive, ++ moderate positive, +++ high positive, - negative						

DISCUSSION

Peste des Petits Ruminants (PPR) is a viral disease in small ruminants that is highly contagious and fatal, which is caused by a Morbillivirus. A recent seroepidemiological study conducted in Syria found a 28% seroprevalence of PPRV antibodies in sheep herds in Hama Governorate, with significantly higher rates among young animals, flocks at markets, and specific districts. These results indicate the continuous circulation of PPRV and the necessity of effective vaccination measures to facilitate control and eradication of PPR (Aldan *et al*., 2025).

The current study demonstrates that humoral immune responses were improved with the addition of chitosan nanoparticles (CSNPs) when assessed by greater and more sustained percent inhibition of c-ELISA. The enhanced antibody responses in the CSNP-adjuvanted samples can be attributed to a series of adequately studied characteristics of chitosan-based nanoparticles. Chitosan is a cationic, mucoadhesive polymer that can extend antigen residence time at mucosal surfaces and thus increase antigen availability for improved penetration into the mucosal epithelium (Mehrabi *et al*., 2018; Mikušová & Mikuš, 2021). In addition, negatively charged biological membranes can interact electrostatically with CSNPs, thereby enhancing uptake by antigen-presenting cells (APCs) and presenting antigens on MHC-I molecules (Mikušová & Mikuš, 2021). These APCs are available in nasal-associated lymphoid tissue (NALT) and or conjunctiva-associated lymphoid tissue (CALT). CSNPs have been reported to stimulate innate immune pathways and enhance recruitment and activation of immune cells at mucosal sites (Collado-Gonzalez & Esteban, 2022), which could contribute to the significant antibody responses observed in this study. Although these mechanisms were not directly investigated here, they provide a biologically plausible explanation for the observed immunogenicity and are consistent with previously published reports (Gao *et al*., 2021; Prego *et al*., 2010; Renu & Renukaradhya, 2020; Shim & Yoo, 2020; Wen *et al*., 2011; Zhu *et al*., 2015)**.**

Nigeria 75/1 and Sungri 96 are two effective live attenuated PPR vaccines that may provide lifetime protective immunity against PPR. These vaccinations are often delivered subcutaneously (Diallo *et al*., 2007). Evidence suggests that the Nigeria 75/1 vaccination was effective in controlling the lineage IV field outbreaks in Morocco in 2008 (Fakri *et al*., 2016) and China in 2007 and 2013 (Liu *et al*., 2018). Although development of a thermostable PPR vaccine is ongoing (Mariner *et al*., 2017), these live attenuated vaccines are currently thermolabile and require a cold chain until they are administered (Diallo *et al*., 2007). A licensed live attenuated influenza virus (Flumist) vaccine that is delivered intranasally has been shown to boost systemic and mucosal protection. Outcomes of pediatric clinical studies evaluating Flumist's effectiveness against an inactivated vaccine indicate that the delivery via the IN vaccine was superior in avoiding influenza (Rhorer *et al*., 2009). Thus, we postulated that, in contrast to the traditional way (s/c) of vaccination, OC or IN vaccination may induce an immunological response very fast.

This study's findings support earlier findings (Emikpe *et al*., 2013; Mahapatra *et al*., 2020) showing that goats may be successfully immunized against PPRV by intranasal administration of live attenuated PPR vaccine (Nigeria 75/1). Clinical observations of the inoculated goats in this investigation revealed that none of the administration techniques raised the rectal temperatures. The outcomes of this research support the findings by Emikpe *et al*. (2013) (Emikpe *et al*., 2013) and Mahapatra *et al*. (2020) (Mahapatra *et al*., 2020) who discovered that intranasal PPR immunization is not associated with low-grade pyrexia, which is commonly observed in the case of invasive vaccination. The preliminary results that vaccine delivery into mucosal surfaces elicits strong systemic immune responses justify the

observed changes in the intranasally vaccinated goat serum PPR-specific IgG responses (Mumin *et al*., 2020; Zhang *et al*., 2007). The same results also concur with the former study by Mahapatra *et al*. (2020), who found the presence of an adequate systemic immune response following intranasal PPR immunization (Igwe *et al*., 2019; Zimmermann & Curtis, 2019).

Clinical outcomes and seroconversion after intranasal delivery of the Nigeria 75/1 PPR vaccine with or without mucoadhesive agents can result in an improved immune response compared to those after subcutaneous vaccination (Ezeasor *et al*., 2021). Similarly, when the gum of *Irvingia gabonensis* is used as an intranasal mucoadhesive, it induces systemic IgG responses and immune modulation, including a decrease in neutrophil-to-lymphocyte ratios (Ezeasor *et al*., 2021). Consistent with these findings, the present study supports the immunological relevance of mucosal PPR vaccination. However, unlike earlier studies relying on natural gums, the use of CSNP in this work may provide additional benefits through enhanced mucoadhesion, improved antigen uptake, and increased interaction with antigen-presenting cells, potentially leading to stronger immune responses (Shim & Yoo, 2020).

This study clearly demonstrates CSNP boosts vaccine effectiveness against PPR. This CSNP-PPR vaccine produced better immune responses (G3 & G4) than the traditional PPR vaccine (G1 & G2) while providing no serious safety concerns in experimental animals. Our study aimed to determine the vaccine-strengthening power of CSNPs when used as an adjuvant for the PPR vaccine. The c-ELISA and VNT analyses showed that CSNP-adjuvanted PPR vaccine (G3 & G4) generated more potent antibodies than the standard PPR vaccine. The IN and OC administration produced more antibodies as compared to the standard local vaccine, which supports CSNP enhancement of the immune response (Renu & Renukaradhya, 2020). In another study, sheep pox mucosal vaccine was designed with CSNPs to using the ionic gelation technique and measured its cytotoxicity and *in vitro* release (Al-Zubaidi *et al*., 2023). The CSNP-based PPR vaccine showed great effectiveness in neutralizing antibodies against PPRV (Mahapatra *et al*., 2020).

It has been demonstrated that CSNP aids in enhancing the immune response to the vaccines through the improvement of antibody and immune cell responses in a wide variety of animal species. As explained by Zhang *et al*. (2019), CSNP boosted the immune response in chickens administered with an inactivated avian influenza vaccine (Zhang *et al*., 2019). Mohamed *et al*. (2018) investigated CSNP in an H5N1 avian influenza vaccine as an adjuvant in ducks and confirmed the enhancement of immune responses with higher neutralizing antibodies (Mohamed *et al*., 2018). It has been reported that nanoparticles, especially Chitosan, improve the efficacy of vaccines, which correlates well with our promising PPR vaccination outcome (Cargnin Faccin & Perez, 2024).

Recent studies further support the immunomodulatory potential of chitosan nanoparticles. El-Qabbany *et al*. (2024) reported that CSNPs, when combined with therapeutic agents in Schistosoma mansoni–infected mice, improved biochemical parameters and enhanced immune responses, including increased IL-10 levels. Although conducted in a parasitic disease model, these findings reinforce the immunological compatibility and adjuvant potential of CSNPs observed in the present vaccine study (El-Qabbany *et al*., 2024).

As a future perspective, the PPR eradication program by the World Organization for Animal Health (WOAH) is focused on global eradication of PPR by 2030, the proposed CSNP-based PPR vaccine formulation can be a good option with inbuilt potential of significant seroconversion and minimized virus shedding through induction of humoral and mucosal immune responses. The World Organization set this target because it had successfully removed rinderpest from the world. The vaccine remains the main strategy for eliminating PPR alongside necessary disease monitoring procedures.

In this global elimination program, our research adds an enormous contribution by making and testing a CSNP-based PPR vaccine. In our research, the animals that had been vaccinated using the CSNP-formulated vaccine had better and sustained immune responses than those that had been vaccinated using the conventional vaccine. This was shown by the results of c-ELISA and VNT, with the CSNP group showing higher values of PI and enhanced neutralizing titers. These results suggest that CSNP-based vaccine delivery has the potential to increase antigen presentation, boost mucosal immunity, and decrease dose-sparing in a multi-dose setting, which is particularly appropriate in a low-resource setting in the field.

The accomplishments of our research meet the criteria of the PPR-GEP because it provides a novel, potentially more thermostable and efficient vaccine platform, which can be added to prevailing vaccination plans. The benefit of the CSNP-based vaccine in terms of immunogenicity can raise the level of vaccination and decreasing the occurrence of the disease, as well as resolving logistical reasons, including dependence on the cold chain. This will resonate well with the goals of the eradication program and assist in creating more sustainable, efficient, and accessible immunization systems, especially in remote or underserved areas. A major limitation of the present study is the small sample size (n = 3 animals per group), which limits the ability to capture inter-individual biological variability and reduces the generalizability of the findings. In conclusion, the findings of our study confirmed that CSNP successfully enhanced the efficacy of the PPR vaccine and provided a promising technique to control PPR outbreaks in small ruminants. Future studies are required to

evaluate the durability of the immune response as well as the potential for scaling the CSNP-adjuvanted vaccine for field applications, evaluation in challenge studies, virus transmission, and shedding.

Conclusion: The findings of this study demonstrates that CSNPs are a potent and effective immune adjuvant for the live attenuated Nigeria 75/1 PPR vaccine when administered via mucosal routes such as Intra-nasal or Intra-ocular. Incorporation of CSNPs in PPR vaccine significantly enhanced and prolonged the immune response in vaccinated goats, as demonstrated by raised white blood cell counts, higher c-ELISA antibody levels, and markedly increased virus-neutralizing titers compared to the conventional vaccine. Particularly, ocular administration of the CSNP-based vaccine elicited strong humoral and mucosal immune responses, highlighting its appropriateness as a practical and field-friendly vaccination approach. The CSNP-adjuvanted vaccine induces strong mucosal immunity through simple and needle-free delivery routes. These approaches offer significant compensations for mass vaccination programs, including reduced logistical constraints, improved compliance, and potential reduction in virus shedding and transmission. Collectively, these findings support the use of CSNPs as a promising adjuvant for PPR vaccination and provide a strategic advancement toward effective, sustainable, and large-scale immunization, reinforcing global efforts aimed at the eradication of PPR by 2030.

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