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Research Article

Utilization of Chitosan as an Immune Response Enhancer in Non-Acidic Vaccine Formulations

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Abstract

The unique properties of chitosan make it stand out as an ideal adjuvant candidate. However, the low solubility of chitosan in non-acidic aqueous media limits its use as an adjuvant. In this study, a method was offered to overcome this limitation, without any structural modifications of chitosan. The proposed method incorporated the preparation of Pickering emulsions via self-aggregation of the chitosan particles. As a model for this preparation, a w/o/w emulsion formulation of the foot-and-mouth disease (FMD) vaccine was utilized. Two vaccine formulations, with and without chitosan, were prepared and compared regarding their physicochemical properties. Laboratory animal (guinea pigs) trials were conducted to reveal the immune response in terms of neutralizing antibody levels. The sera were collected from two animal groups and tested for neutralizing antibody levels using a homologous virus neutralization test (VNT). Statistical analyses of the test results were performed at a 5% significance level using Quasi-Least Squares Regression (QLS). The incorporation of chitosan into the w/o/w vaccine formulation did not compromise the physicochemical properties of the emulsion. Statistical analysis reveals that the presence of chitosan enhances the antibody response. The effect of time and group on the neutralizing antibody titer levels (NATL) was found to be significant ($p < 0.05$). Our results suggest that the limitation of the use of chitosan as an adjuvant could be overcome by the Pickering emulsion preparation approach, without a need for further structural modifications. The presence of chitosan in such a vaccine formulation could enhance the immune response.

Keywords: Adjuvant, Chitosan, Pickering emulsion, W/O/W emulsion, Quasi-least squares regression

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Introduction

Vaccination is one of the most effective methods of protection against infectious diseases (Sorgi et al 2020). The purpose of vaccination is to form a strong, protective, and long-lasting immune response to the antigen used. However, a weak immune response occurs when inactive microorganisms are used alone as antigens in a vaccine formulation (Alexander and Brewer 1995). To ensure that the immune response remains at a protective level as long as possible, substances called "adjuvants" are needed (O'Hagan et al 2020). Adjuvants have been an important milestone in the continuous research for safer vaccines with longer lasting and higher levels of immunity (O'Hagan et al 2020). They can act as means of transport of antigens to target immune system cells or as immune stimulants (Reed et al 2009, Reed et al 2013). They can also affect the nature of the response that will occur, determining the occurrence of Th1 or Th2 cells (Mahakapuge et al 2015). Using an adjuvant also reduces the amount of antigen per dose required for a protective immune response level (Reed et al 2013, Boyle et al 2007). An ideal adjuvant should not be toxic, should not show side effects, and should be easily available and cheap while making the maximum contribution to the immune response (Petrovsky and Aguilar 2004). On the other hand, their use is often limited because of their toxicity or their side effects (Shi at al 2019). To overcome these limitations and to increase their effectiveness, one approach is to combine different types of adjuvants

in the same formulation (Mount et al 2013, Garg et al 2017, O'Hagan et al 2020).

Chitin, the second most abundant biopolymer after cellulose, is a natural polymer found in crustaceans, insects, and the cell walls of some bacteria and fungi. Chitosan, on the other hand, is a linear polysaccharide composed of randomly distributed glucosamine and N-acetylglucosamine units linked by β-(1→4) glycosidic bonds (Rinaudo 2006). It is obtained by N-deacetylation of chitin (β-1,4-poly-N-acetyl-D-glucosamine) in alkaline conditions (Kumar 2000, Kumari et al 2015). Apart from the diverse uses of chitosan in different fields (Prashanth and Tharanathan 2007, Choi et al 2016), its adjuvant properties have also received attention (Seferian and Martinez 2000, van der Lubben et al 2001, Şenel and McClure 2004, Li et al 2021). Chitosan is biocompatible, biodegradable, and non-toxic (Rinaudo 2006), and it has been shown to stimulate both humoral and cellular immune responses (Zaharoff et al 2007). These properties make chitosan an ideal adjuvant candidate (Şenel and McClure 2004, Li et al 2021). However, its solubility in aqueous media depends on pH and is very low at pH values of 6.5 (the pKa ≈ 6.5) and above (Liu et al 2005). At pH values below 6.5, the amino groups of chitosan are protonated, and it becomes soluble in water. Although there are vaccine studies, such as for nasal vaccines (Çokçalışkan et al 2014, Günbeyaz et al 2010), this feature limits its use as an adjuvant in vaccines with neutral or alkaline pH values. One can modify the chitosan structure by adding water-soluble groups, and this is a strategy to address the insolubility of chitosan in a non-acidic aqueous environment (Prashanth and Tharanathan 2007). However, additional procedures are needed to modify the chitosan structure, and the possible toxicity and side effects caused by these procedures as well as the new chitosan structure must be evaluated as well.

Emulsions are colloidal dispersions of two immiscible liquid phases: the oil phase and the water phase (Schramm 2005). Since they are thermodynamically unstable systems, emulsifiers and stabilizers are included in the emulsion formulations (Schramm 2005). Pickering emulsions—named after S. U. Pickering, who reported them in 1907 (Pickering 2001)—are defined as emulsions stabilized by solid particles. The chitosan-based Pickering emulsions have gained attention given their biocompatibility and high stability (Sharkawy et al 2020). Recently, Pickering emulsions have started to gain attention as vaccine adjuvants, combining the features of emulsions and particulate (Xia et al 2018, Peng et al 2020, Xia et al 2020). Although the action mechanism of emulsions as an adjuvant is not completely clear, the depot effect and the slow release of the antigens are among the most probable ones. The protective effect against the enzymes from degradation might further contribute to the immune response (Aucouturier et al 2001).

Foot-and-mouth disease (FMD) is a contagious viral disease of cloven-hoofed animals. The disease is accompanied by fever, vesicles in the mouth, lameness, and sudden death among young animals (de Los Santos et al 2018). Not only the economic losses but also the restrictions on international trade caused by FMD make it one of the most feared diseases (Diaz-San Segundo et al 2017). Routine vaccination is one of the main and most effective strategies to combat the disease in endemic countries. Vaccines can also be used to mitigate the spread of the virus before the culling of infected animals in case of re-emergence in disease-free countries (Orsel and Bouma 2009, Dar et al 2013). The FMD vaccines are formulations with whole inactivated virus antigens and an adjuvant. To keep the antigen intact, the pH value should not be out of the 6.5–9.0 range (Grubman and Baxt 2004). Neutral and slightly over-neutral pH values are ideal. These conventional vaccines have been used since 1950s, however, to sustain protective immunity, repeated vaccination should be done (Diaz-San Segundo et al 2017). Mineral oil adjuvants are widely used for the preparation of FMD vaccines as emulsions. Montanide ISA 206 (Seppic, France) is a frequently used mineral oil adjuvant, which, blended with an aqueous FMD inactive antigen phase, leads to the water-in-oil-in-water (w/o/w) multiple emulsion FMD vaccine (Cao 2014).

The longitudinal data consists of repeated measurements of each observation unit at different time points. In instances where there are missing values in the measurements and the measurements are conducted at unequal time intervals, the utilization of traditional methods adversely affects the statistical analyses. The quasi-least squares regression (QLS) method is a specialized approach developed for analyzing such data (Hedeker and Gibbons 2006, Agresti 2007, Kim and Shults 2010, Shults and Hilbe 2014). In this method, working correlation structures such as the Markov correlation structure are employed to construct a model. This structure was developed for measurements with unequal time intervals, taking into account the actual measurement times (Ziegler 2011, Shults and Hilbe 2014, Wang 2014).

The aim of this study is to investigate the possibility of using chitosan, without it being modified structurally, in a formulation of non-acidic emulsion vaccines. For this purpose, as a model vaccine, a w/o/w emulsion–type FMD vaccine was used. The formation of

Pickering emulsions with chitosan was mimicked, and the colloidal particles of chitosan were obtained via the self-aggregation method. The effect of chitosan on the immune response was evaluated by conducting laboratory animal trials.

Material and Methods

The animal experiments were done according to EU Directive 2010/63/EU with the approval of the Ankara FMD Institute's Local Ethics Committee (Approval No: 15/06-1).

Preparation of the vaccines

As an oil adjuvant and for the preparation of the w/o/w emulsion, Montanide™ ISA 206 BVG (Seppic, France) was used. Chitosan was medium-viscosity chitosan (Fluka, 28191), and the FMD vaccine strain ASIA-1/TUR/15 (Sindh-08) inactivated antigen suspension was provided by the Ankara FMD Institute. The antigen suspension was a BHK-21 cell-cultured and harvested virus culture, clarified by filtration, inactivated by binary ethyleneimine (BEI) (3 mM BEI concentration, at 26°C for 24 hours), cross-flow concentrated, and PEG 6000 purified. All the other chemicals used were in analytical grade.

The vaccines were prepared in a laminar airflow biosafety cabinet ensuring a sterile environment. The chitosan solutions were filtered through 0.8 µm Millex AA MF-Millipore MCE Membrane filters, and the oil adjuvant and antigen suspensions were filtered through 0.2 µm filters. All the solutions and suspensions were used at 30°C after incubation. The amount of the antigen per dose was adjusted to be equal in both vaccines (2.0 µg/mL). The type of emulsion was confirmed by drop tests (Aucouturier et al 2001) and conductivity measurements (Aucouturier et al 2001). The viscosity measurements were performed at the 15 rpm shear rate and the temperature of 25.0 (±0.02)°C. The Heidolph Hei-Torque 100 homogenizer was used to prepare the emulsion formulations, the Brookfield DV3-T rheometer was used for viscosity measurements, and conductivity measurements were performed using the WTW Inolab Terminal Level 3 conductivity meter. Images of the emulsion were taken using the Leica DM750 microscope and imaging systems with the LAS V4.3 software.

Preparation of the experimental FMD vaccine without chitosan

The antigen suspension was added into the oil adjuvant of equal volume at the rate of 2 mL/min under the 1,000 rpm constant stirring speed of the homogenizer. Mixing was maintained for an additional 10 minutes. The type of emulsion was confirmed by a drop test. The outer continuous water phase of the emulsion was confirmed by measuring the conductivity (5.40 mS/cm).

Preparation of the experimental FMD vaccine containing chitosan

The final vaccine formulation with chitosan was obtained in two steps (Figure 1).

The first step was the preparation of two different emulsions. To prepare the first emulsion, a 1.0% (m/v) chitosan solution in 1.0% (v/v) acetic acid was added into the oil adjuvant of equal volume at a rate of 2 mL/min under the 1,000 rpm constant stirring speed of the homogenizer. Mixing was maintained for an additional 10 minutes. The type of the obtained w/o/w emulsion was confirmed by a drop test. The initial pH value of the emulsion was 4.00 (±0.02). Then the pH was adjusted to 7.15 (±0.05) by adding a NaOH solution (50% m/v) in volumes of 100 µL under gentle stirring. The viscosity was 40.3 mPas. To prepare the second emulsion with the antigen suspension instead of the chitosan solution, the same procedure (excluding the pH adjustment step) was applied. Namely, the antigen suspension was added into the oil adjuvant of equal volume at the rate of 2 mL/min under the 1.000 rpm constant stirring speed of the homogenizer, and mixing was maintained for an additional 10 minutes. The type of the obtained w/o/w emulsion was confirmed by a drop test. The pH of the emulsion was 7.66 (±0.02), and the viscosity was 18.6 mPas.

The second step was blending these emulsions in equal volumes. The emulsion with the antigen was added to the emulsion containing chitosan at the rate of 10 mL/min under constant stirring (1.000 rpm). Mixing was maintained for an additional 30 minutes. A drop test confirmed that the type of the obtained final emulsion was not changed. The pH value was 7.46, and the viscosity was 28.2 mPas. The conductivity value of 3.50 mS/cm confirmed that the outer continuous phase of the emulsion was a water phase. The concentration of chitosan in the final vaccine formulation was calculated to be 0.25% (m/v).

Laboratory animal experiments

The experimental FMD vaccines (1.0 mL/dose) were administered to six-month-old Dunkin-Hartley male guinea pigs via the subcutaneous route (Habiela et al 2014). The animals, possessing similar characteristics, were randomly divided into two study groups. Each group consisted of 10 animals. One group was vaccinated with the vaccine formulation containing chitosan, and the other group was vaccinated with the vaccine formulation without chitosan. In this two-group experimental study, the sample size was determined by a priori power analysis using the G*Power software (3.1.9.7 version, Kiel, Germany). In this power analysis, the power of the test was 80%, the effect size was 30%, and the level of significance was 5%. Blood samples were taken from the lateral saphenous vein of

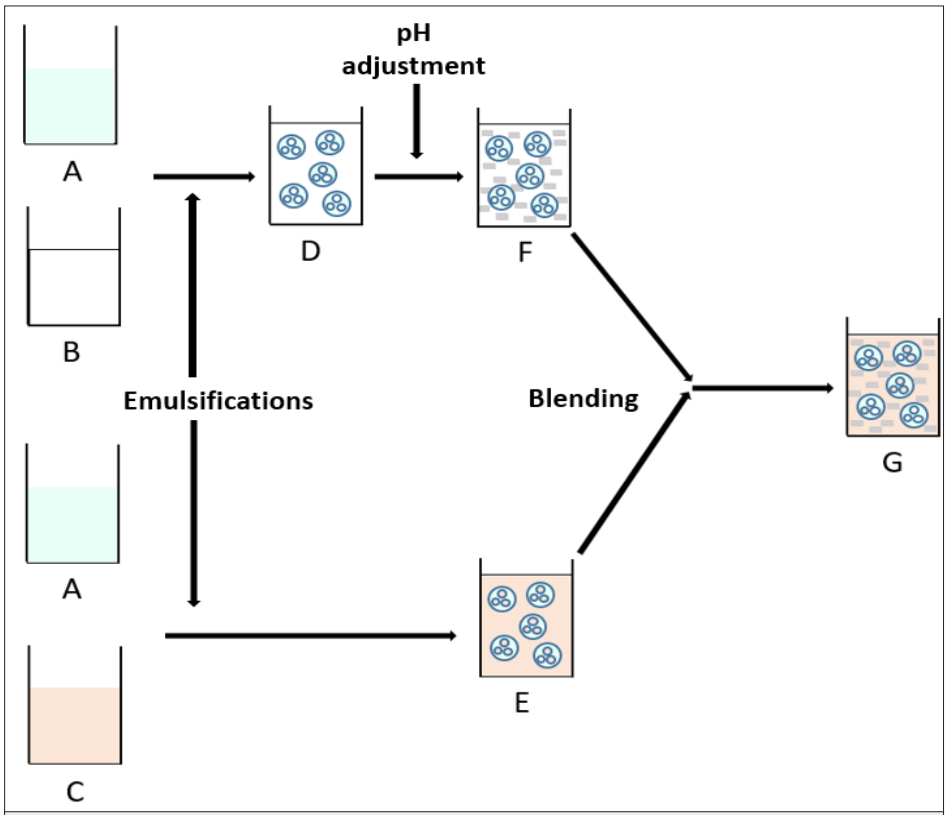

Figure 1. Preparation of the experimental FMD vaccine containing chitosan; A: Oil phase- Montanide ISA 206, B: Water phase- 1 % (m/v) chitosan solution in 1 % (v/v)Acetic acid, C: Water phase – the antigen mixture, D: w/o/w emulsion – chitosan solution (water phase) + Montanide ISA 206 (oil phase), E: w/o/w emulsion – antigen mixture + Montanide ISA 206, F: Pickering emulsion – chitosan (prespitation in the water phase) + Montanide ISA 206 (oil phase) G: Pickering emulsion – chitosan (solid phase) + Montanide ISA 206 (oil phase) + antigen mixture (water phase)

the animals with 23G needle under general anesthesia to measure the neutralizing antibody titer levels (NATL) by a homologous virus neutralization test (VNT). Samples were collected pre-vaccination and on days 7, 14, 28, 60, 120, 180, and 220 post-vaccination. The sera were stored at −20°C until the test.

Homologous virus neutralization test

The VNT was performed according to the OIE manual (OIE 2011). The guinea pig serum samples were heated for 45 minutes in a water bath adjusted to 56°C to inactivate the complement. The serial dilutions of the sera, which started from 1:16, were made in 96-well cell culture plates using a Glasgow minimum essential medium (Biochrom GmbH, Germany). The dilutions were made by a dilution robot (Viaflo 96, Integra Biosciences, Switzerland) to minimize pipetting errors. One hundred TCID50 (Asia-1/TUR/15) homologous virus suspensions were added to the wells containing the diluted sera. One-hour incubation was performed in a 37°C and 5% (v/v) CO2 incubator. Following incubation, a BHK-21 C-13 cell suspension containing 600,000 cells/mL was added to all wells. After 48 hours, the cells were stained with crystal violet dye to visualize the cytopathic effect (CPE). The last reciprocal serum dilution that inhibited the CPE formation in 50% of the wells was accepted as the titer of neutralizing antibodies in the serum.

Statistical evaluation of the results

According to the compiled data, there are repeated measurements on two groups of 10 animals; the measurements were made seven times, and the measurement time intervals were not equal. In addition, measurements could not be made on some animals at some times. This led us to conclude that we needed to analyze a data set that included measurements with unequal time intervals and missing measurements (missing observations rate: 10.71%). A two-way analysis of variance (ANOVA) with repeated measures cannot be used in the analysis of such unbalanced data; thus, data analysis was performed using the QLS method. For this purpose, the QLS regression model was established using the Stata (version 14.1) software. In this model, a Markov correlation structure was used as the working correlation structure, which allows use for measurements with unequal time intervals by considering the actual times of

Table 1. The results of the regression model based on the QLS method

Response Variable	Working Correlation Structure	Coefficient, Error, p, Confidence Interval (95%)		Constant	Time	Group
NATL	Markov	Regression Coefficients		10.1820	0.0057	−0.7655
		Standard Error		0.4437	0.0017	0.2633
		p Value		0.0000	0.0010	0.0040
		Confidence Interval (95%)	Lower limit	9.3124	0.0023	-1.2815
			Upper limit	11.0515	0.0090	-0.2493

For time × group interaction; regression coefficient: 0.0026, p-value: 0.4360 and lower limit and upper limit of the confidence interval: -0093 and 0.0040 respectively NATL: Antibody titer level obtained according to the VNT.

the measurements. In this regression model, the NATL response (dependent) variable was included according to the vaccine strain used in the VNT. In addition, groups were defined as the "group" variable and measurement times as the "time" variable as independent variables in this model. Statistical decisions were made using a significance level of 0.05. The effect of the interaction term (time × group) on the dependent variable was also examined (Table 1). Y is the response variable, while β is the regression coefficient, and the model can be written as follows: $Y(NATL)=\beta 0 +\beta 1\times time+\beta 2\times group$.

Results

Physicochemical evaluations for the emulsions

Both vaccines, without (Figure 2) and with chitosan (Figure 3), were w/o/w multiple emulsions. The presence of chitosan did not change the type of the emulsion. The chitosan was successfully self-assembled into colloidal particles by increasing the pH to 7.15 (±0.05). The particles were dispersed on and among the droplets of the emulsion (Figure 3). The pH values for both vaccine formulations were within the range of 7.40–7.60. The final viscosity of the formulation with chitosan (28.2 mPa·s) was with no significant difference in practice comparing with that of the conventional vaccine formulation (21.0 mPas). Both formulations were evaluated for stabilization over time by visual inspection, drop tests, and conductivity measurements and were found to be stable for at least one year at 4°C.

Statistical evaluations for neutralizing antibody response

Using NATL as well as the time and group variables, a regression model was established according to the QLS method, taking into account the correlation between measurements. The results obtained according to these models are given in Table 1. As shown by this table, the group and time effects on NATL were statistically significant ($p<0.05$). In addition, the study data used to obtain these results are given in Table 2.

Discussion

Although chitosan is insoluble in a neutral aqueous environment, the approach and the method presented in this study as well as the obtained results show that its utilization in vaccine formulations as an adjuvant without any further structural modifications is possible.

As a delivery system model, a traditional w/o/w emulsion formulation of the FMD vaccine was utilized. The formation of Pickering emulsions was imitated by allowing the chitosan particles to self-aggregate into the emulsion. While mimicking the formation of Pickering emulsions in this way, the primary goal was not to stabilize the already stable emulsion but to enable the incorporation of chitosan into the existing formulation without compromising stability (Figure 2 and Figure 3). The viscosity of the formulation with chitosan (28.2 mPas) was with no significant difference from that of the conventional vaccine formulation (21.0 mPas). Both formulations were evaluated for stabilization over time by visual inspection, drop tests, and conductivity measurements and were found to be stable for at least one year at 4°C. Therefore, it could be concluded that the presence of chitosan at 0.25% (m/v) concentration did not adversely affect the stability of the emulsion.

Based on the action mechanism of adjuvants, they can be separated into two categories, namely immune enhancers and delivery systems (Reed et al 2009, Reed et al 2013). The delivery systems carry the antigens to the cells of the immune system and protect them from degradation (Alexander and Brewer 1995, Reed et al 2013). The adjuvants acting as immune enhancers can directly activate immune cells through specific receptors such as toll-like receptors (TLRs) (De Gregorio et al 2013).

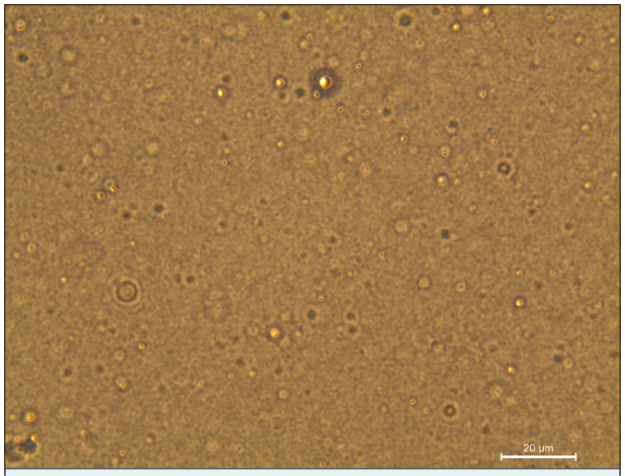

Figure 2. Microscopy image of the w/o/w emulsion FMD vaccine without chitosan. The image was obtained under a light microscope; water droplets can also be seen within the oil droplets typical of w/o/w emulsions.

Chitosan gained attention as a vaccine adjuvant given its unique physicochemical and biological properties (Li et al 2021), and the action mechanism of chitosan was thought to be as an immune enhancer (Zaharoff et al 2007). In this study, the two categories of adjuvants were combined into one formulation. Chitosan could be an adjuvant acting as an immune enhancer (Zaharoff et al 2007), and the emulsion as an adjuvant acting as a delivery system. The results of this study revealed that the presence of chitosan in the w/o/w emulsion type of FMD vaccine enhanced the immune response. In such a formulation, chitosan might have the primary task to stimulate the immune system, and together with the emulsion might have positive synergistic effect on overall immune response.

Various vaccination studies have been carried out with Pickering emulsions. Different substances have been used to create vaccines with Pickering emulsion formulations such as particulate alum (Xia et al 2018). It was reported that the Pickering emulsions could enhance the antigen uptake and presentation with antigen-presenting cells (Xia et al 2018, Peng et al 2020, Xia et al 2020). It was also demonstrated that aluminum with Emulsigen D, an oil-in-water emulsion, produces a rapid neutralization antibody response against FMD in dairy goats (Park et al 2014). Contrary to most substances with adjuvant activity, chitosan is non-toxic and biocompatible. Moreover, its immune-enhancing and penetration-enhancing properties (van der Lubben et al 2001, Sonaje et al 2011) make it a candidate for an ideal adjuvant. Taking into account the relative toxicity of alum (Tomljenovic and Shaw 2011), the utilization of chitosan could be suggested as a potentially safer and more effective strategy.

In the particular case of FMD vaccines, mineral oils have been used as adjuvants for several decades. Montanide™ ISA 206 is one of the most common oil adjuvants for this purpose (De Gregorio et al 2013). However, the robust immune response needs repeated vaccinations. To overcome this problem, various additives have been experimented with to fortify the vaccine (de Los Santos et al 2018). Among these substances, alum (Park et al 2014), cytokines (Nagaraj et al 2017), and TRL agonists (Ren et al 2011) can be mentioned. Apart from being non-toxic and biocompatible, considering the results of this study, chitosan could be suggested as an alternative as well.

Conclusion

In this study, it was shown that chitosan, without any further modifications, can be used as an adjuvant in non-acidic environments by mimicking the preparation of Pickering emulsions of chitosan via controlled self-aggregation. According to the study, chitosan can be incorporated into the conventional FMD vaccine formulation, and its presence in this formulation elicits a significantly enhanced immune response. This study could serve as a framework for further studies with different antigens, in which chitosan might be used with more biocompatible emulsion formulations that do not necessarily show immune-stimulating properties but need only carry and protect the antigens, leaving the immune stimulation task to chitosan. Hence, the alternatives for the utilization of biocompatible and non-toxic substances for emulsion design with chitosan could be broadened. The obtained results are encouraging for the utilization of chitosan in other available or new emulsion formulations of vaccines.

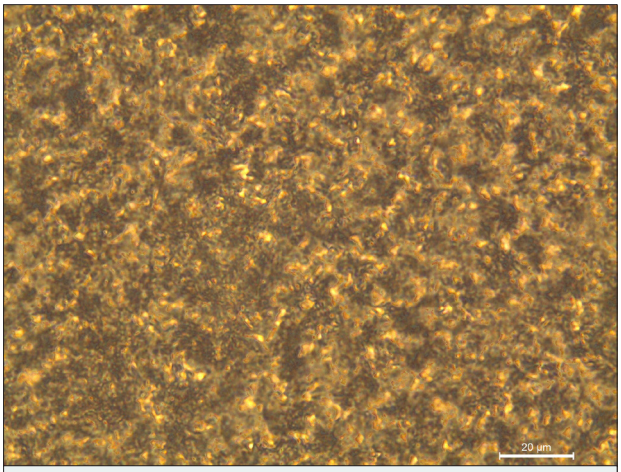

Figure 3. Microscopy image of the w/o/w emulsion FMD vaccine with chitosan. The image was obtained under a light microscope; previously seen droplets are not seen because of the precipitated and dispersed chitosan particles.

Table 2. Obtained NATL values according to groups and measurement times (study data)

		Post-vaccination measurement times and NATL values b,c (NATL/day)						
Group[a]	ID	7	14	28	60	120	180	220
1	1	8.61	9.00	10.01	11.00	10.58	11.00	10.58
1	2	7.00	8.61	9.00	11.58	9.58	10.01	8.58
1	3	8.00	9.59	10.01	12.58	12.58	10.58	9.58
1	4	8.00	7.00	9.00	11.00	11.00	11.00	.
1	5	9.00	7.00	9.00	10.01	7.58	.	.
1	6	9.00	9.00	10.01	11.58	11.00	11.00	10.01
1	7	8.00	8.00	10.01	11.58	.	.	.
1	8	7.00	7.58	10.01	12.00	10.58	10.01	.
1	9	7.58	8.00	.	.	.	.	.
1	10	9.00	8.61	10.01	11.58	.	10.01	.
2	11	8.00	7.58	9.00	9.58	11.00	9.00	9.58
2	12	9.00	9.00	10.01	11.00	9.00	9.58	9.00
2	13	9.00	9.59	10.01	11.00	9.00	11.58	11.00
2	14	8.61	7.58	8.00	7.58	9.58	9.58	10.58
2	15	6.00	7.00	9.59	8.58	7.00	9.58	7.58
2	16	8.00	8.00	10.01	10.00	10.00	11.00	9.58
2	17	8.00	8.00	10.01	11.00	8.00	8.51	7.00
2	18	8.00	7.58	9.00	8.00	.	11.00	8.58
2	19	8.00	8.00	8.61	9.00	9.58	10.58	8.58
2	20	7.58	9.00	9.59	10.01	11.00	11.00	10.01

[a]Group 1: Neutralizing antibody levels obtained from animals vaccinated with vaccine formulation containing chitosan; Group 2: Neutralizing antibody levels obtained from animals vaccinated with vaccine formulation which does not contain chitosan
[b]NATL: Antibody titer level obtained according to the VNT test
[c](.): No measurement data available

Declarations

Competing Interests
Author declare that there are no conflicts of interest related to the publication of this article.

Funding
The study was carried out in the facilities of the Ministry of Agriculture and Forestry, Institute of Foot and Mouth Disease and with the Institute's resources.

Availability of Data and Materials
The data that support the findings of this study are available on request from the corresponding author.

Ethical Statement
This study was carried out with the approval of the Ankara FMD Institute's Local Ethics Committee (Work Registration Number: 1551/440 and FMD Institute's Local Ethics Committee Decision Number 15/06-1; Decision Date:25.06.2025; The ethics committee approval document number 93369491.HDYEK-604-6 and date of the document: 30.06.2015).

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Author Contributions
Motivation/Concept: TT, CC; Design: TT, CC; Control/Supervision:

TT, CC, EA; Data Collection and Processing: TT, CC; Analysis and Interpretation: TT, CC, EA; Literature Review: TT, CC, EA; Writing the Article: TT, CC, EA; Critical Review: TT, CC, EA

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