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Research Article

Designing a Vaccine Platform composed of Phage encapsulated with Chitosan Nanoparticles Targeting Pathogenic Viruses

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A B S T R A C T

Introduction: The current study is aimed at investigating the feasibility of designing a novel platform using chitosan-coated bacteriophage for vaccination against pathogenic viral agents. The SARS-CoV-2 spike protein was used as a model in this study. The novel coronavirus SARS-CoV-2 is responsible for the COVID-19 disease and relies on ACE2 as its obligate receptor to enter cells. The receptor-binding domain (RBD) of the virus is an important epitope for neutralising activity and is efficiently produced. To improve immunisation, *E. coli* phage particles are being used as vaccine delivery vehicles, and chitosan (CS) is a promising candidate for use as an adjuvant/ carrier in vaccine delivery.

Methods: The process included the preparation of antigens, isolation of bacteriophage, encapsulation with chitosan (CS-NPs) to form CS-NPs loaded with phage (phage-CS-NPs), loading with RBD protein, characterisation of phage by TEM, while that of chitosan (CS-NP) and encapsulation of phage-CS-NP by FTIR spectra, followed by measurement of released RBD protein by HPLC technique, vaccination of rabbit, and then detection of antibody by competitive ELIZA.

Results: Nanoparticle adjuvant of phage encapsulated with chitosan and loaded with RBD antigen has shown to induce good titre of anti-RBD IgG antibodies after being injected in rabbits for three doses and 2 weeks intervals in between, similar to the effect of alum, but with less toxicity than alum.

Conclusion: It has been shown that using nanoparticles complex adjuvant with alum adjuvant was more effective in boosting humoral immunity than if they were used separately.

Keywords: Vaccine Platform, Nanoparticle Adjuvant, CS-NPs, Vaccine Delivery Vehicles, Phage-CS-NPs, SARS-CoV-2, RBD

Introduction

SARS-CoV-2 is a member of the Coronaviridae family. It is a single-stranded, positive-sense RNA virus that causes COVID-19.[1] It mainly infects the lungs' bronchioles and alveoli.[2] Its genetic material is made up of RNA, and it also contains a phospholipid envelope that houses the phosphorylated N protein.[3] The virus envelope also has S proteins, along with the membrane M protein and envelope E protein.[4] S protein facilitates the entrance of viral particles into host cells. First, it binds to a host receptor using the S1 subunit's receptor-binding domain (RBD). It then uses the S2 subunit to fuse the viral and host membranes.[5] Therefore, it is crucial to define the RBD in the SARS-CoV-2 S protein for the synthesis of antiviral drugs, neutralising antibodies, and vaccines that prevent virus attachment.[6] It has been determined that the human ACE2 (hACE2) protein is an important target for the S protein of the SARS-CoV, which allows the virus to adhere to the host's epithelial cells.[7] Here, the ACE2 receptors act as the virus' entrance point into the body. The virus multiplies while impairing other organs that express ACE2, including the heart, kidneys, blood vessels, and digestive tract.[8] Virus-induced immunological reactions trigger antibody-mediated defences. When the B cells differentiate into plasma cells, which then produce antibodies tailored to a viral antigen, the T cells provide assistance. An antibody with a neutralising nature is effective in completely preventing the virus from entering host cells to limit infection. It also plays a very strong protective role in the latter stages of infection and inhibits infection relapse.[9] The development of nano-design platforms for vaccines has seen notable progress, leading to the creation of highly effective strategies for generating strong immune responses.[10] The RBD of the SARS-COV-2 spike protein region is a prime candidate for vaccine development; nevertheless, with extensive efforts to create RBD-based vaccines, the RBD subunit's low immunogenicity still makes it difficult to use it as a vaccine candidate. Researchers suggest modifying RBD to produce a larger antigen-carrier complex in order to boost immunogenicity. However, the manufacture and confirmation of the recombinant modified antigen would be delayed as a result of this modification, which would complicate the overall RBD structure.[11] The idea of employing phages as nanomedicine platforms for creating vaccines has been investigated extensively due to their distinctive biological properties. For the creation of phage-displayed vaccines or phage DNA vaccines, the entire phage particle can be used.[12]

One of the most popular encapsulating agents, chitosan, is a cationic natural polysaccharide that is produced by deacetylating the naturally occurring polymer chitin. It is known for its biocompatibility, biodegradability, and low toxicity.[13] Its potential use as a vaccination adjuvant and a drug delivery mechanism has been studied.[14] The immune system priming and booster adjuvants known as aluminium salts are frequently employed in vaccines. Adjuvants made of aluminium have a proven track record, but there is a risk of over vaccination and there have been reports of negative and toxic effects.[15]

Materials and Methods

Study Design

The present study was an experimental work, designated for *in vivo* immunising of phage loaded with SARS-CoV-2 spike protein. The laboratory work was conducted at the Central Public Health Laboratory, Ministry of Health, while the *in vivo* part was done at the Veterinary Drugs Research and Production Center, Ministry of Industry and Minerals. The protocol for the preparation of antigens included isolation of bacteriophage, encapsulation with chitosan (CS-NPs) to form CS-NPs loaded with phage (phage-CS-NPs), and loading with RBD protein, along with the characterisation of phage and chitosan (CS-NP) by methods of TEM and FTIR spectra, and measurement of released RBD protein by HPLC technique. This was followed by the vaccination of the laboratory animal and the detection of specific anti-RBD IgG antibodies by competitive ELIZA. The research duration spanned from June 2022 to April 2023.

Methods

Isolation of Bacteriophages

According to the procedure[16] used in this study, primary bacteriophages were isolated and multiplied. Clean test tubes were used to collect various crude samples for bacteriophage isolation from 50 ml of sewage water. Filtered sewage was mixed with *Escherichia coli* bacterial stock, which was then combined with salt of magnesium (SM) buffer before being incubated at 37 °C overnight. The mixture was then passed through a (0.20 - 0.22 µ) syringe filter. The suspension was moved and mixed with an equal volume of previously prepared SM buffer. A spot lysis test on nutrient agar was used to screen bacteriophages, while a top-layer plaque assay was performed to measure the concentration of bacteriophages in suspension.

Encapsulation of Bacteriophage with Chitosan Nanoparticles (CS-NPs)

To prepare the bacteriophage nanoparticles, a suspension of 250 µl bacteriophages (108 PFU/ml) was added to 800 µl of the chitosan counterion solution and then mixed with 0.7% sodium deoxycholate. The solution was added dropwise to 40 ml of neutralised chitosan solution containing 1% Pluronic® F68 as a dispersive agent. The mixture was stirred at a low rate for an hour, and the resulting nanoparticles were collected by centrifugation, washed twice with PBS (pH 7.4), and then resuspended in a 4% trehalose solution.

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Finally, the nanoparticles were stored in glass vials at 4 °C.[17]

Loading of Phage-CS-NPs with BSA and RBD

Tris-HCl buffer solution (0.02 M) was prepared in 1 ml of distilled water. BSA, the standard protein and RBD were mixed separately with 0.02 M Tris-HCl buffer solution and a pH of 8.5 was reached. CS-NPs were mixed with 0.02 M Tris-HCl buffer solution and a pH of 5.5 was reached. Then equal volumes (500 µl) of BSA and RBD were mixed separately with 500 µl of CS-NPs and incubated overnight at 37 °C.[18] The binding affinity was determined by the Bradford method.[19]

Characterisation of CS-NPs and Phage-CS-NPs

FTIR spectra of pure CS-NPs powder and lyophilised phage-CS-NPs complex were obtained on TENSOR27-Pruker Fourier transform infrared spectrometer. The samples were spectral scanned within the range of 400-4000 cm^{-1}, while the morphology and characterisation of free bacteriophage and phage-CS-NPs was examined under TEM aria SUPRA 55 VP with STEM detector (Germany), Electron microscope unit, College of Pharmacy, University of Basrah.

The *in vitro* Release Study of BSA and RBD

The assay was used to determine the amount of protein released from CS-NPs that bounded with BSA or RBD. BSA was used prior to RBD as a standard protein. The study was carried out according to the protocol described by Rahimzadeh et al. with some modifications.[20] Different known concentrations of BSA (0.25, 0.5, 1, 2, 4, and 8 µg/ml) were measured by HPLC to determine the BSA standard curve. 1 ml of CS-NPs was loaded with 8 µg of BSA. 10 ml of suspension was prepared in a glass tube which was mixed well and incubated at 37 °C overnight with continuous stirring. Samples (500 µl) were collected at predetermined time points of 2, 4, 6, 8, and 10 days to obtain 5 aliquots of suspension that were centrifuged. Each supernatant was moved to another clean tube and was measured by HPLC to determine the rate of concentration of BSA released from the complex. The same procedure was followed using RBD. According to different concentrations of RBD in different solutions, the optimum solution was chosen depending on the concentration of RBD antigen that was released from the complex over time. The HPLC analysis was conducted using a SYKAM-German model on C18 column (250 mm x 4.6 mm, 5 µm particle size) at ambient temperature. The purpose was to measure the consistent release of free RBD protein from RBD-loaded phage-CS-NPs, with UV detection at 272 nm.

Binding of RBD Protein with Adjuvant Aluminium Hydroxide

The aluminium hydroxide was used as a standard adjuvant for comparison with the new adjuvant design phage-CS-NP. The purified recombinant RBD protein was mixed with aluminium hydroxide at a ratio of 1:20 and was incubated overnight at 35 °C.[21]

Immunisation Programme of Rabbit

15 rabbits of type Albino, male, aged 1-1.5 years and 2-2.5 kg in weight were divided into 4 groups as shown in Table 1.

Table 1.Study Groups of Rabbits according to the Type of Adjuvant

Group No.	Type of Immunogen	Concentration of RBD in a Single Dose (µg)	Volume of Single Dose (µl)	Number of Doses	Route of Admission
1.	RBD + phage-CS-NPs	30	600	3	IM
2.	RBD + alum	30	600	3	IM
3.	RBD + phage-CS-NPs + alum	30	600	3	IM
4.	Phage-CS-NP	-	600	3	IM

Immunoassay of Anti-RBD

Blood samples (5-10 ml) were obtained by cardiac puncture. The concentration of anti-RBD IgG was determined in serum using a competitive enzyme-linked immunosorbent assay (ELISA) kit manufactured by Elabscience, USA following the manufacturer's instructions.

Statistical Analysis

Statistical analysis was done using Microsoft Excel (version 9) and SPSS (version 26).

Ethics Statement

The animal study was reviewed and approved by the IRP of Al-Nahrain University, College of Medicine.

Results

To determine whether a bacteriophage sample may infect *E. coli*, a spot lysis experiment was carried out. Lytic bacteriophages were observed as shown in Figure 1.

The titre of bacteriophage was determined wherein serial dilutions of bacteriophage were made starting from 10^{-1} up to 10^{-9}. The calculation of the plaques was based on the optimal dilution that produces the greatest number

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of reasonable plaques with distinct borders and without overlapping one another, which was 39 at a dilution of 10^{-6} as shown in Figure 2.

The titre was determined to be 3.9 X 10^9 pfu/ml.

Phage titre was calculated for a dilution of 10^{-6}:

Pfu/ml = pfu counted/dilution

= 39 X 10^6 per 100 µl

= 3.9 X 10^8 per 1000 µl

Figure 1.A Central Clear Zone of Lytic Bacteriophages based on Spot Assay

Figure 2.Top Layer Assay showing Plaques with Clear Margins and without Merging with each other at a Dilution of 10^{-6}

According to the TEM result, bacteriophages were classified as Caudovirales due to their tails. Podoviridae is one of the families that belong to this order. Its members have a short, non-contractile tail[22] as shown in Figure 3.

The FTIR spectrum of chitosan showed a range of frequencies for a number of active groups band at 3268 cm^{-1} related to the OH group of chitosan, while the peak at 2920 cm^{-1} belonged to the C-H aliphatic of chitosan. Another band appeared at 1617 cm^{-1} related to C=O of the amide group of chitosan. Other frequencies appeared as shown in Figure 4. New bands related to the new complex (phage-CS-NPs) appeared in the spectrum. The first band was seen at 3676 cm^{-1} due to the OH of chitosan. This band showed a change in shape and position as a result of the bonding of chitosan with phage. The band at 1693 cm^{-1} referred to the carbonyl group (C=O). A new band appeared at 1651 cm^{-1} related to the azomethine group (C=N) of amino acid of the polypeptide of phage protein, as shown in Figure 5.

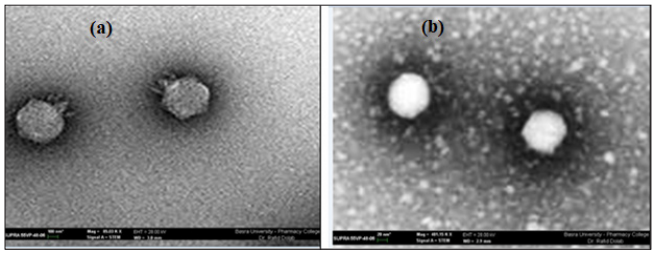

Figure 3(a).Podoviridae Coliphage (b) Phage encapsulated with CS-NP

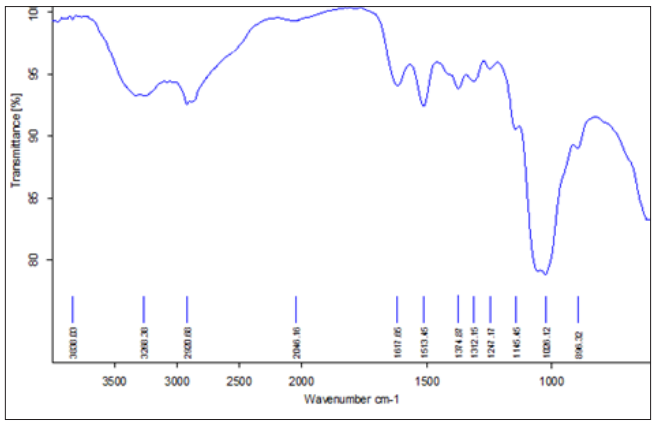

Figure 4.FTIR Spectra of Pure Chitosan Nanoparticles (CS-NPs)

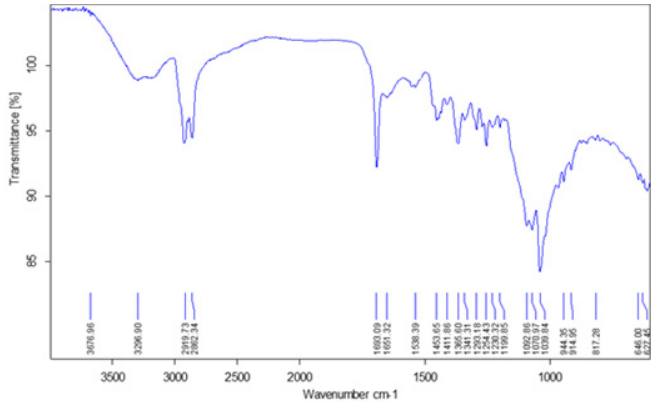

Figure 5.FTIR Spectra of Bacteriophage-loaded Chitosan (Phage-CS-NP)

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The concentrations (µg/ml) of released BSA from the suspension of phage-CS-NPs loaded with BSA (8 µg/ml) were measured by HPLC for 10 days (Table 2).

The results of released RBD concentrations with time for all 4 samples have been shown in Table 3.

The concentrations of RBD antibodies in the study groups (Table 1) were measured in rabbit serum by cELISA and have been shown in Figure 6.

control group of phage-CS-NP showed close mean levels of antibodies ($p > 0.05$).

Discussion

It is highly important to develop an effective vaccine against the SARS-CoV-2 virus, which is the main cause of the respiratory COVID-19 pandemic disease. As an essential step during an infection, the RBD of the spike protein is utilised by SARS-CoV-2 to interact with the

Table 2.Concentrations (µg/ml) of Released Free BSA during 10 Days

Duration Time	Day 2	Day 4	Day 6	Day 8	Day 10
Concentration (8 µg/ml)	0.31	0.4	0.52	0.37	0.26

Table 3.Concentrations of Released Free RBD with Time

Suspension Tube Number	RBD Concentration (µg/ml)	RBD pH Value	Phage-CS-NPs pH Value	Concentrations of Free RBD (µg/ml)	
				After 3 days of incubation	After 7 days of incubation
1.	8	6	10	0.08	0.2
2.	2	6	10	0.5	1.1
3.	8	10	5.5	1.6	3
4.	2	10	5.5	1.0	1.6

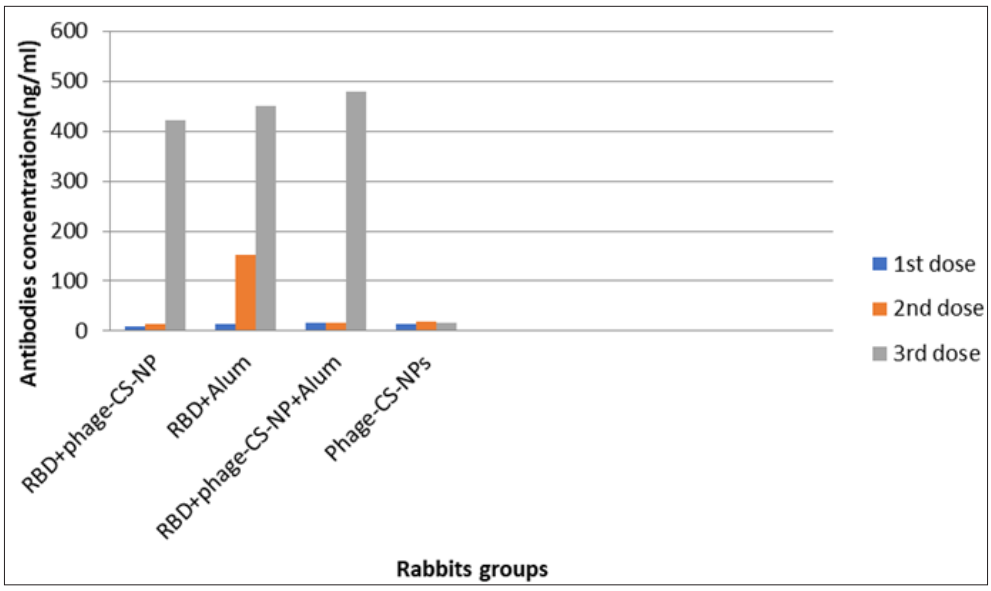

Figure 6.RBD Antibody Concentrations of Rabbit Groups

After the first dose, there was no significant difference in mean values of antibody levels among different tested groups or between control and tested groups ($p > 0.05$). However, after the second dose, the antibody level in the RBD-alum group was much higher than the other groups ($p < 0.0001$), while no significant difference was observed among other groups after this dose. After the third dose, the highest antibody level was seen in RBD + phage-CS-NP + alum, followed by RBD-alum, and RBD-NP, while a

receptor angiotensin-converting enzyme 2 (ACE2) on host cells.[23,24]

A powerful functional antibody response is induced by a recombinant vaccine that contains the spike protein's RBD in vaccinated mice, rabbits, and non-human primates.[25]

Based on its high availability, simplicity of separation, and accessibility of reliable assays for its identification, the *E. coli* bacteria were chosen for this study's bacteriophage

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isolation. Since *E. coli* is a gut bacterium, grows best at body temperature (37.4 °C), and is simple to work with in the laboratory, scientists initially chose to work with it. Additionally, because *E. coli* has a rapid growth rate, experiments involving it can be completed quickly, easily, and affordably.[26]

A spot lysis assay was used to detect lytic bacteriophages that infect *E. coli* isolated from sewage water samples. Sewage water was thought to be the finest source for isolating *E. coli* bacteriophages because of its harsh environment, which allows the bacteriophages to withstand extreme physical conditions and produce plaques that are distinctive and higher in titre than those of other bacteria. The spot assay was performed to check the isolated phages' capacity to kill bacteria.[27]

Small-sized plaques with distinct zones of lysis were formed, and the lytic bacteriophage titre was determined using a two-fold overlay technique. Serial dilutions from 10^{-1} to 10^{-9} were used to measure the phage titre. After overnight incubation at 37 °C, plaques were counted and the titre of the isolated phage was determined to be 3.9 X 10^{9} pfu/ml.

A plaque assay was used to purify and measure the titre of the isolated phages, and a spot test assay was used to identify their host range.[28]

Since encapsulation is a coating procedure to improve the stability of bioactive substances, chitosan was used in this study because of its special qualities. It is important to note that many authors have used this technique like Yadav et al.,[29] Sharifi-Rad et al.,[30] and Murugesan and Scheibel.[31]

Bacteriophages were classified as Caudovirales according to TEM results because of their tail. According to the shape of the tail, Caudovirales were split into three families: Myoviridae, which were bacteriophages with long contractile tails were distinguished from Siphoviridae, which had long non-contractile tails, and Podoviridae, which had short non-contractile tails.[22] The bacteriophage in this study had a short non-contractile tail. Based on its properties and according to this classification, it was assumed that it could belong to the Podoviridae family.

The results of chitosan bands and new bands related to the new complex (phage-CS-NPs) that appeared in the FTIR spectrum indicated that the spectrum of the complex had strong binding between chitosan and phage. The researchers in a study used FTIR spectroscopic analysis to investigate the functional groups of chitosan involved in bacteriophage-loaded chitosan microspheres. They compared the absorption intensity of pure chitosan microspheres with bacteriophage-loaded chitosan microspheres and found that the latter had higher absorption intensity at 2922.98 cm^{-1}, 2860.50 cm^{-1}, and 1649.59 cm^{-1}. The increase in absorption intensity of the $–CH_2$ group and C=N double bond was attributed to the glutaraldehyde cross-linking reaction. Moreover, the FTIR spectrum of bacteriophage-loaded chitosan microspheres showed significant changes in the shape and frequencies of the bands, confirming the involvement of glutaraldehyde.[32]

The estimated concentrations of released BSA and RBD in this study were measured by HPLC. The *in vitro* study showed that BSA was released from the phage-CS-NPs complex in a relatively slow constant pattern and the same occurred with RBD in 4 different solutions. These findings showed that the encapsulated phage with CS-NP could act as a good adjuvant for the *in vivo* immunisation trial in a laboratory animal. The liberation of minor concentrations of RBD may trigger the immune response to produce specific immunoglobulin for that antigen. The recombinant RBD protein has been demonstrated to be an effective antigen and an innovative adjuvant needed for the efficient induction of adaptive immunity. Therefore, chitosan cationic nanocarriers were selected as effective adjuvants for mucosal and intramuscular vaccination of the recombinant RBD vaccine of SARS-CoV-2 in order to increase the efficiency of the vaccine and adjuvant-mediated promotion of humoral and cellular immunity.[33]

Regarding *in vivo* immunisation, the rationale behind selecting rabbits as a source of target-specific antibodies was their clear benefits over mice. They can elicit a significant immune response against antigens or epitopes that are poorly immunogenic or tolerated in mice because they make higher affinity antibodies than mice.[34]

The first three groups of rabbits showed anti-RBD IgG titre after injection of three doses of 30 µg RBD adjuvanted with phage-CS-NPs, alum, and a combination of phage-CS-NPs with alum, intramuscularly, with two weeks' interval between them, while the last group injected with CS-NPs alone was used as control.

The results of antibody response showed that there was no significant difference in the levels of antibodies between different tested groups and the control group after the first dose. However, after the second dose, the level of antibodies in the RBD-alum group was significantly higher than in other groups, while there was no significant difference among other groups. After the third dose, the highest level of antibodies was observed in the RBD + phage-CS-NP + alum group, followed by the RBD-alum group, and the RBD-NP group. The control group of phage-CS-NP showed similar levels of antibodies after all three doses with no significant difference in the level of antibodies. It is worth noting that the group receiving a combination of the two adjuvants showed a better-boosting effect compared to when they were administered separately. In a previous study, rabbits were intramuscularly immunised with a 20 µg dose of RBD adjuvanted with either liposomes or alum, on days 0 and 21.

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On day 21, the post-immune serum revealed the presence of anti-RBD IgG, and on day 42, there was a boosting effect that caused the antibody levels for liposomes to be almost 10-fold higher than those for alum.[35] In another study, a combination of alum and CpG adjuvants resulted in an 80-fold increase in anti-RBD neutralising antibody titres in mice compared to the use of alum alone.[36] Furthermore, Meena et al. found that using RBD entrapped in polylactic acid (PLA) nanoparticles along with aluminium hydroxide adjuvant led to 9-fold higher immune responses compared to RBD adsorbed on aluminium hydroxide alone, which is a commonly used adjuvant in human immunisation.[37]

Conclusion

This study aimed to create a new method for delivering vaccines, and it used chitosan nanoparticles encapsulating phages as an adjuvant. These nanoparticles were found to be both safe and effective in generating a protective immune response by producing specific anti-RBD IgG antibodies in rabbits when injected with a coating of RBD protein. The neutralising antibodies' titre of nanoparticle adjuvant was similar to some extent to that of the standard adjuvant, alum, and the combination of both had a greater effect.

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Conflicts of Interest: None

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