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ENHANCEMENT OF IMMUNOPOTENCY OF EQUINE HERPES VIRUS-1 VACCINE USING NANOPARTICLES

By

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ABSTRACT

Chitosan and calcium phosphate nanoparticles (CN and CaPN) either individually or encapsulating inactivated Equine herpesvirus-1 (EHV-1) were prepared. Morphology and size of CN and CaPN before and after loading with the virus were studied. There was no cytotoxicity of CN and CaPN observed on VERO cells by two successive subcultures of inoculated cells. Infectivity test revealed that blank EHV-1and EHV-1encapsulated withCaPN(EHV-1- CaPN) titers were higher than EHV-1 encapsulated in CN (EHV-1-CN). Immune responses of (EHV-1- CN) and (EHV-1-CaPN) vaccines were evaluated by immunization of both mice and horses. In mice, (EHV-1-CN) vaccine in ratio of 1:1 induced high immune response followed by commercial vaccine then (EHV-1-CaPN) vaccine in ratio of 1:1. By measuring the IFN-γ level in mice serum, it was found that, the highest significant level was induced in mice inoculated with EHV-1-CN at ratio of 1:1 followed by commercial EHV-1 vaccine comparing with other groups. Challenge of inoculated mice followed by virus re-isolation revealed complete viral clearance at 5th dpc in EHV-1-CN (1:1) whereas achieved at 6th, 7th, & 9th dpc in commercial vaccine, EHV-1-CN (2:1) and EHV-1-CaPN (1:1) respectively. Immunogenicity of horses vaccinated with (EHV-1-CN) vaccine (1:1) was monitored for 28 WPV. We concluded that using of chitosan nanoparticles as a novel adjuvant in preparing of EHV-1 vaccine could induce protective levels of immunity in vaccinated animals.

Keywords:

Chitosan nanoparticles; Calcium phosphate nanoparticles;EHV-1 vaccine encapsulation.

INTRODUCTION

Equine herpesvirus-1 is an important ubiquitous viral pathogen that induce sporadic or storms of abortion in pregnant mares, early neonatal death, respiratory disease in young foals, equine myeloencephalopathy (EHM), and chorioretinopathy **(Patel and Heldens 2005, Hussey *et al.* 2013 and Wagner *et al.* 2015)**. EHV-1 reactivates during times of stress and shed again,with all clinical manifestations which may be seen during recrudescence **(Lunn *et al.* 2009)**. The recent increase in incidence of morbidity and mortality in neurological EHV-1 infection suggests a change in virulence of the virus **(Henninger *et al.* 2007 and Perkins *et. al.*2009)** and prompted the USDA to classify Equine Herpes virus Myeloencephalopathy (EHV-1-EHM) as an emerging disease **(USDA-APHIS 2011).** Vaccination remains the most cost-effective biomedical approach to control infectious diseases in livestock using either live attenuated or inactivated vaccines **(Liu *et al.* 2017).** Inactivated vaccines require the presence of strong adjuvant to stimulate higher immune responses **(Paillot *et al.* 2008)**. A wide choice of chitosan delivery systems such as aqueous dispension, gel, sponges and nanoparticles have been shown to carry antigens **(Arca *et al.*2009).** The unique charater of chitosan nanoparticles could exhibits more superior than chitosan as adjuvant. CN has been synthesized as drug and vaccines delivery system due to their bioahesive, biocompatibility, biodegradability and penetration enhancement properties. It mostly taken up by phagocytic cells inducing strong systemic and mucosal immune response **(Van der lubben *et al.* 2001 and Zhu *et al.* 2007).** Calcium phosphate nanoparticles (CaPN) adjuvant found to be effective in vaccines against intracellular pathogens in which an antibody immune response alone is insufficient for protective immunity **(Sahdev *et al.* 2013)**. In a murine model, a novel composite of CaPN adjuvant was more potent than alum, elicited little or no inflammation at the site of administration, induced high titers of immunoglobulin G2a (IgG2a) and neutralizing antibody, that facilitated a high percentage of protection against HSV-2 and Epstein-Barr virus (EBV) infection **(He *et al.* 2002**). Both CN and CaPN have been previously tested as mucosal adjuvant showed potential results **(Boonyo *et al.* 2007 and Rauw *et al.* 2010).** IFN-γ synthesis is one of the most widely used markers of cell mediated immunity (CMI) to measure responses to herpes viruses in human and animals **(Paillot *et al.* 2005, 2006 and Breathnach *et al.* 2005).** IFNγ promotes CMI by increasing viral peptides presentation by antigen presenting cell (APC) and promoting the development of T helper 1 (Th1) lymphocytes. IFN-γ is a key factor in the development of a type-1 response involving

cytotoxic T lymphocytes (CTL), which ultimately leads to the destruction of virus infected cells **(Paillot *et al.* 2008)**. In horses, the up regulation of mRNA expression of IFNγ, IL-2, IL-4, IL-8, and IL-10 has been shown after experimental infection or vaccination against EHV-1 as reported by **(Hussey *et al.* 2011, Coombs *et al.* 2006, Pusterla *et al.* 2006 and Wagner *et al.* 2010).** Thus, the present work was designed to study the preparation and the efficiency of chitosan and calcium phosphate nanoparticles as adjuvants with Equine herpes virus-1 vaccine to increase the immune response and protect animals from EHV -1 infection.

MATERIAL AND METHODS

Virus:

EHV-1 was isolated from Egypt in 2003 and 2007 by **Warda (2003)** and **Saleh *et al.* (2009)**. It was supplied by Equine Viral Disease Research Depart., Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo.

Antisera:

1- Rabbit anti-EHV-I serum was kindly obtained by Dr. Jannet Wellington, Research Fellow, Department of Biological Science, Macquarie Univ., NSW, and Australia. It was used for virus identification.

2- Locally prepared rabbit anti EHV-1 hyper immune serum by **Warda *et al.* (2005)**. It was used as a positive serum control in serological tests and in detection of EHV-1by ELISA.

Antigen for serology:

Purified antigen was prepared from the EHV-1 (locally isolated strain) and propagated on VERO cell according to **Meredith *et al.* (1989).**

Mice:

1- Groups of Swiss albino mice 4-6 weeks old were used to evaluate the stability and potency of the prepared vaccines.

2- Groups of Swiss albino pregnant mice were used to test the safety of the prepared vaccines.

African green monkey kidney cells (VERO):

They were maintained and grown in Eagle's minimum essential media supplemented with 10% newly born calf serum, antifungal, penicillin sodium 100 IU/ml and streptomycin 100 mg/ml. They were used in propagation of vaccinal strain.

Embryonated Chicken Eggs (ECE):

Specific pathogens free embryonated chicken eggs (SPF-ECE), 11-13 days old were used in virus re-isolation from challenged mice.

Binary ethyleneimine (BEI):

0.1M binary ethyleneimine (Aldrich chemical Co.LTD) were used as virus inactivator according to **Bahnemann (1990)** and **Saleh (2006).**

Mice IFN-γ ELISA Kit (Quantikine):

Used in estimation of total IFN-γ levels in inoculated mice.

Preparation of nanoparticles:

Chitosan was extracted from marine shrimp shells with a degree of deacetylation of 85% as determined by potentiometric titration and molecular weight of 2122 kDa as determined from the intrinsic viscosity measurement according to **Morsi *et al.* (2017).** Sodium tripolyphosphate (TPP) was purchased from (Sigma - Aldrich). Calcium chloride, dibasic sodium phosphate and sodium citrate were purchased from (Sigma - Aldrich) to be used in preparation of calcium phosphate nanoparticles.

Preparation of chitosan nanoparticles (CN):

According to the principle of ionic crosslinking, nanoparticles can be formed by intra and inter molecular crosslinking between positively charged chitosan and negatively charged TPP. Chitosan solution (1% w/v) was prepared by dissolving chitosan with sonication in an aqueous solution of 2% acetic acid until the solution was transparent and the pH was adjusted at 5.4. TPP was dissolved in deionized water at the concentrations of (1% w/v) according to **Zhao *et al.* (2012)**. Chitosan nanoparticles were prepared by dropping TPP solution to the chitosan solution with the ratio of 1:5 (v/v) under magnetic stirring at room temperature overnight. CN was separated by centrifugation at 10,000 rpm for 30 min at 4˚C and the supernatant was discarded then reconstituted in PBS to its initial volume.

Preparation of calcium phosphate nanoparticles:

Calcium phosphate nanoparticles were formulated as described by **He *et al.* (2002)** with some modifications. Briefly, 12.5 mM calcium chloride was added to equal amount of 12.5 mM dibasic sodium phosphate then 10 mM sodium citrate in ratio of 10% was slowly added drop by drop to control pH 7.2. These chemicals were mixed together and stirred using a magnetic stirrer overnight at room temperature.

Preparation of inactivated EHV-1:

A) Master seed of EHV-1(local isolate VEp2) was grown on VERO cells for another three passages to prepare vaccine seed virus (VEp5), then identified by serum neutralization test (SNT) using reference anti-EHV-1 serum.

B) From the vaccine seed virus, a vaccine stock viral fluid was prepared by another passage on VERO cells and the bulk virus harvested fluid was kept at -70˚C tell the titration and sterility test were done.

C) Vaccine stock viral fluid having virus titer not less than 7 log10 $TCID_{50}$/ml as metioned by **Mayr *et al.* (1978)**. It was inactivated by 0.008M BEI at 37˚C for 24 hours as described by **Saleh (2006).**

Encapsulation of inactivated EHV-1 with chitosan nanoparticles:

Chitosan nanoparticles coupled with Equine herpesvirus-1 (EHV-1-CN) were prepared using an ionic cross-linking method according to **Zhao *et al.* (2012)** with some modifications. Inactivated EHV-1 was added drop by drop to 1% chitosan solution prepared as previous, in ratio of (1:1 v/v) and (2:1 v/v) under magnetic stirring for 2 hours. Subsequently, TPP solution was added in the same manner as mentioned before. EHV-1- CN was separated by centrifugation at 10,000 rpm for 30 min at 4°C and the supernatant was discarded. Then reconstituted in PBS to the initial volume and used for immunization protocol.

Encapsulation of inactivated EHV-1 with calcium phosphate nanoparticles:

Inactivated EHV-1 was added to12.5 mM calcium chloride in final ratio (1:1 v/v) and (2:1 v/v) then complete the steps of CaPN preparation as mentioned before according to **He *et al.* (2002).** EHV-1-CaPN was separated by centrifugation at 10,000 rpm for 30 min at 4°C and the supernatant was discarded then reconstituted in PBS to the initial volume.

Evaluation of encapsulation Efficiency:

The encapsulation Efficiency of EHV-1 - CN and EHV-1–CaPN were determined by separating the EHV-1-CN and EHV-1- CaPN encapsulated nanoparticles from the aqueous medium by centrifugation at 10,000 rpm for 30 min at 4°C. The amount of free viral antigen in the supernatant was determined by ELISA (96 well ELISA plate coated with locally prepared rabbit anti EHV-1 hyper immune serum).

Particles morphology and size characterization:

Morphology and size of CN and CaPN before and after loading with Equine herpes virus-1 were measured using Jeol JEM-1400 transmission electron microscopy (TEM) (Japan) at an acceleration voltage of 80 KV. The prepared materials were dispersed in ethanol and sonicated for 2 min for better dispersion and to prevent particle agglomeration on the copper grid. One drop of the suspension was spread onto a carbon-coated copper grid, which was then dried at room temperature for TEM imaging.

In vitro cytotoxicity of nanoparticles:

Cellular compatibility of nanoparticles of chitosan and calcium phosphate was investigated by direct contact test according to **Günbeyaz *et al.* (2010)** using VERO cells which was well established culture system for vaccine production. 24-well cell culture plate was cultivated at density $3x10^3$ cells/well and incubated for 24 hours in a humid chamber at 37°C under 5% CO_2 atmosphere. Then 100 µl from undiluted CN and CaPN as well as diluted (1:1 and 1:2) nanoparticles in MEM were inoculated into monolayer cells after discarding the growth medium. After incubation for one hour, media was removed and fresh MEM free serum was added 100µl/well. Cell morphology and viability was checked microscopically for 72 hours.

Infectivity test:

Both EHV-1, EHV-1- CN and EHV-1 - CaPN were inoculated onto monolayer VERO cells. After incubation for two hours, fresh MEM free serum was added and incubated at 37°C till cytopathological effects (CPE) were achieved then subjected for titration, and $TCID_{50}$ was calculated according to **Reed and Muench (1938).**

In-process control:

It was performed according to **OIE (2015)**.

1- Identity test.

SNT was applied using reference anti-sera against EHV-1.

2- Sterility Test.

Samples were taken from the final product as well as the virus fluid before inactivation process and tested on nutrient agar medium,Sabouraud dextrose agar medium, Thioglycolate medium (Oxford,England) and PPLO(broth) medium for bacterial,fungaland mycoplasma contaminations.

3- Safety Test.

The residual infective virus activity was examined by inoculation of inactivated virus fluid on:

A) VERO cells which was incubated at 37ºC for seven days with daily observation. Additionally blind passage was done to ensure complete virus inactivation **OIE (2015)**.

B) Group of Swiss albino mice at late stage of pregnancy were inoculated intranasal (I/N) by 45µl of inactivated virus fluid.

C) Another four groups of Swiss albino pregnant mice at late stage of pregnancy were inoculated subcutaneous (S/C) by two doses from each four final vaccine formulations (EHV-1- CN at ratio of 1:1 and 2:1) and (EHV-1 - CaPN at ratio of 1:1and 2:1) with one week interval (0.2 ml/dose) according to **Awan *et al.* (1991) and Madkour *et al*. (2016)**.

All experimental mice were kept under observation until parturition.

4 - Potency of the prepared vaccines.

4 -1- In mice.

Five groups of Swiss albino mice 4-6 weeks old (20 mice/group) were inoculated subcutaneously (0.2ml/dose) with 5 different vaccine formulations as shown in (Table 1). The 6th group was kept as unvaccinated control. After one week, each group inoculated with booster dose of the same vaccine **(Saleh 2006)**. 14 days later, all groups were bled to collect serum samples from the tail vein, EHV-1 antibodies were estimated by indirect ELISA according to **Sugiura *et al.* (1997)** and **Madkour *et al.* (2016)** and total IFN-γ levels were estimated using mice IFN-γ ELISA Kit (Quantikine) according to manufacture protocol. All groups were challenged I/N by 45 μl of living EHV-1 ($10^6 TCID_{50}$ /ml). Three mice from each group were sacrificed at each interval time (3rd, 5th, 7th, 9th and 10th days post challenge). Ten percent of liver and lung suspension of sacrificed mice were inoculated onto chorioallantoic membrane (CAM) of ECE to detect the role of the vaccine in reduction of virus multiplication.

4-2 -In Horses.

Three healthy susceptible horses with low antibodies titer against EHV-1 (<4 neutralizing antibody) were intramuscularly I/M vaccinated by two doses with four weeks interval (2ml/dose/horse) from the prepared vaccine with formula EHV-1- CN (1:1) which gave a good result in mice inoculation. Another three horses were vaccinated I/M with EHV-1 commercial vaccine and other group of horses kept as unvaccinated control. Blood samples were collected from jugular veins of horses in each group every 2 weeks for monitoring humoral immunity up to 28 weeks post vaccination (WPV) on separated serum samples using indirect ELISA (96 well ELISA plate coated with locally isolated EHV-1).

Keeping quality of the prepared vaccines:

The prepared vaccine formula EHV-1- CN (1:1) was stored at 4 ºC for time interval (3, 6 and 12 months) after preparation. Groups of Swiss albino mice (5 mice/group) were inoculated with the prepared vaccine. 14 days later, serum samples were collected and evaluated by ELISA to determine the vaccine stability.

Ethics Statement:

Care of laboratory animals and experimentation were conducted in accordance with animal ethics guidelines.

Statistical analysis:

Data were statistically analyzed using IBM SPSS® version 19 software for personal computer (2010). Means were compared by one-way ANOVA ($P<0.05$).

RESULTS AND DISCUSSION

Despite an extensive use of inactivated virus vaccines in many countries over decades, less respond to vaccination was recorded **(Foote *et al.* 2002)** and abortion storms are still being reported **(VanMaanen *et al.* 2000).** Therefore, it is important to improve the whole inactivated EHV-1 vaccines by using novel adjuvant such as chitosan and calcium phosphate nanoparticles to increase the immune response and protect animals from EHV -1 infection. Successful vaccination against EHV-1 requires both humoral and cellular immune responses. Thus, different immune responses have to be stimulated by vaccines to treat the different aspects of the disease induced by EHV-1. The humoral immune responses, stimulated by vaccination is generally quantified by high titers of virus neutralizing antibodies, will reduce epithelial infection and virus shedding by neutralizing extracellular viruses. However, the cellular immune response assessed by IFN-γ synthesis which can directly block pathogen replication, activate macrophages for EHV-1 elemination and activate NK cells to lyse infected cells **(Paillot *et al.* 2008)**.

Preparation of nanoparticles:

Both of the prepared chitosan nanoparticles and calcium phosphate nanoparticles either alone or after encapsulating EHV-1 using an ionic cross-linking method were produced with good morphology, showing spherical poly-dispersed nature as revealed by transmission electron microscope (TEM) Fig. (1a and b) and Fig. (2 a and b), respectively. The particles size of the prepared chitosan nanoparticles as well as calcium phosphate nanoparticles increased after loading with EHV-1. This enlargement in the nanoparticles size confirms the EHV-1 encapsulation.

Encapsulation efficiency:

Coupling of EHV-1 with either CN or CaPN efficiency were estimated by applying indirect ELISA on supernatant fluids after centrifugation of the prepared vaccines. The results proved absence of free viral antigens in the supernatant fluids and this indicates complete encapsulation.

In vitro cytotoxicity:

On using VERO cells, at 72 hours post inoculation of CN and CaPN, no toxic effects were observed (i.e. no changes in cell morphology or detachment of cells from surface) either in undiluted or diluted (1:1& 1:2) inoculums. In addition, successive blind subcultures of inoculated cells were achieved without any alteration of cell morphology which was an indicator of cell viability. This result comes in parallel with **Sayin *et al.* (2008), Janvikul *et al*. (2007) and Günbeyaz *et al*. (2010)** who concluded that loaded microparticles and gel formulations of chitosan were shown to maintain cell viability and antigen integrity.

Infectivity test:

EHV-1 infectivity was evident by CPE, 72 hours post infection (PI), whereas cells infected with CN and CaPN coupled with EHV-1 consumes longer incubation (80 hours PI). The virus titer was 8.5 $log_{10}TCID_{50}$ /ml in blank EHV-1 and (EHV-1 - CaPN) while in (EHV-1-CN) the virus titer decreased to be 8 $log_{10}TCID_{50}$ /ml. indicating that the CN affected on virus titer. This result is in coincidence with **Wang *et al.* (2012)** who reported that chitosan effectively reduced the infectivity of feline calicivirus FCV-F9 and bacteriophages MS2 and phiX174.

Potency of the prepared vaccines in mice:

Immune response of the prepared EHV-1 encapsulated in CN and CaPN vaccines were evaluated by immunization of both mice and horses.

Concerning mice inoculation (Table 1), the obtained result revealed that, (EHV-1- CN) vaccine induced higher immune response than (EHV-1- CaPN) and commercial vaccine, with mean ELISA antibodies titer 2332.34 ± 29.87 and 2236.67± 80.07 in ratio (1:1 and 2:1) , respectively. The results coincidence with **Volkova *et al.* (2014)** who found that inactivated Newcastle disease virus (NDV) vaccine in combination with chitosan or Cap increased the antibody titers in blood and mucosal samples of chickens when compared with the administration of NDV antigen only and observed also that chitosan based vaccine has higher antigenic and protective activity than vaccine contain CaPN following challenge with ND virus, considering chitosan a potential adjuvant in poultry. Also **Wen *et al.* (2011)**, showed that when mice were administered subcutaneously twice with a dose of 25 µg ovalbumin (OVA) dissolved in saline containing CN (12.5, 50 or 200 µg), the serum OVA-specific IgG, IgG1, IgG2a, and IgG2b antibody titers were significantly enhanced. On the other hand, the groups of mice inoculated by EHV-1- CaPN vaccines 1:1 and 2:1 showed the least antibody

levels between groups, where the registered mean ELISA titers were 515.34 ± 49.08 and 179.00 ± 12.42 respectively. However, **He *et al.* (2002)** concluded that mice immunized with HSV-2 contained CaPN induced specific mucosal IgA as well as systemic neutralizing antibodies that protect animals against live infection. Regarding to IFN-γ level in sera of inoculated mice (Table 1), revealed that, the highest significant IFN-γ level was in group 1 (G1) of mice which vaccinated with EHV-1-CN nanoparticles in a ratio of 1:1 followed by group 5 (G5) which vaccinated with commercial EHV-1 vaccine. Previous study by **Zhang *et al.* (2003)** demonstrated a high frequency of cells secreting pro inflammatory tumor necrosis factor-alpha (TNF-alpha), interferon gamma (IFN-gamma), and interleukin 4 (IL-4) in the lungs of mice in response to infection with EHV-1 KyA or RacL11 or immunization with recombinant viral glycoprotein D (rgD). Also, **Kim *et al.* (2016)** showed that EHV-1 immunization increased the expression of IFN-γ and 16 antiviral interferon-stimulated genes (ISGs). Thus, murine IFN-γ inhibited EHV-1 infection of murine alveolar macrophages and protected mice against lethal EHV-1 challenge, suggesting that IFN-γ expression is important in mediating the protection elicited by EHV-1 KyA immunization. To prove the potency of different vaccine formulations, challenge with EHV-1 was achieved in inoculated mice followed by virus re-isolation from lung and liver (Table 2). Complete viral clearance was observed at 5th dpc in mice inoculated with EHV-1-CN (1:1) whereas achieved at 6th, 7th & 9th dpc in groups inoculated with commercial vaccine, EHV-1-CN (2:1) and EHV-1-CaPN (1:1) respectively. In case of mice inoculated with EHV-1-CaPN (2:1) vaccine and the control un-inoculated groups, the EHV-1 was still re-isolated until 10th dpc.

Immunization of horses with (EHV-1- CN):

Evaluation of immunogenicity of horses after immunization by EHV-1-CN (1:1) and commercial vaccines (Table 3) revealed that, at 2WPV ELISA antibody titers were increased significantly in group I comparing with the commercial vaccine (group II). After booster dose of vaccination, significant increases in antibody titers were recorded, reaching their peaks at 8WPV. (1760.34 ± 30.02 and 1548.67 ± 25.1) in EHV-1- CN and commercial vaccines, respectively, as recommendation by **OIE (2015).** The antibody titers were decreased gradually till the end of the experiment at 28 WPV.

Keeping quality of the prepared vaccines:

The prepared vaccines could be stored for 1year at 4˚C without losing their immunizing potency when investigated in mice.

Based on aforementioned data, it can be concluded that:

- EHV-1-CN vaccine induced higher immune response than EHV-1-CaPN vaccine especially at ratio (1:1) in immunized mice and horses.
- The highest significant IFN-γ level was obtained in mice vaccinated with EHV-1-CN vaccine at ratio of 1:1 followed by commercial vaccine.
- Horses could be vaccinated with two doses of EHV-1- CN vaccine (4 weeks interval) and revaccinated after 5 months to maintain the protective levels of EHV-1 antibodies in their sera.

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Table (1): ELISA antibody titers and IFN-γ levels 2 weeks post second vaccination of mice with different vaccine formulations.

IFN-γ pg/ml	ELISA	Groups (G)
45.13 ± 1.30 A	2332.34 ± 29.87 A	G1 EHV-1: CN(1:1)
35.97± 1.94 aB	2236.67± 80.07 B	G2 EHV-1: CN (2:1)
29.40 ± 0.15 abC	515.34± 49.08 abC	G3 EHV-1 : CaPN (1:1)
30.97 ± 0.23 abD	179.00± 12.42 abcD	G4 EHV-1 : CaPN (2:1)
43.30 ± 0.67 bcdE	1536.67 ± 40.96 abcd	G5* commercial vaccine
27.77 ± 0.19 abde	Negative	G6 control un-inoculated
The mean difference is significant at 0.05 levels. Small letters against capital letters in the same column indicate significant difference between groups (using ANOVA test) *G5 EHV-1 inactivated vaccine adjuvanted with Alum gel (commercial vaccine).		

Table (2): Virus Re-isolation from mice inoculated with the prepared vaccines after challenge.

Mice Groups	Virus re-isolation in day post challenge(dpc)						
	3rd	4th	5th	6th	7th	9th	10th
G1 EHV-1:CN(1:1)	50%	25%	-ve	-ve	-ve	-ve	-ve
G2 EHV-1:CN(2:1)	80%	75%	50%	30%	-ve	-ve	-ve
G3 EHV-1: CaPN (1:1)	100%	100%	75%	40%	30%	-ve	-ve
G4 EHV-1: CaPN (2:1)	100%	100%	75%	50%	30%	20%	10%
G5 commercial vaccine	75%	50%	50%	-ve	-ve	-ve	-ve
G6 control un-inoculated	100%	100%	80%	75%	50%	30%	10%

Table (3): Immune response of horses vaccinated with inactivated EHV-1vaccine encapsulated with chitosan (EHV-1- CN 1:1) by ELISA.

Time of sampling	ELISA antibody titers in vaccinated horses		
	Group I	Group II	Group III
Pre vaccination	59.34±20.18	46.00 ± 6.25	48.34 ± 4.41
	1st dose of vaccine		
2wpv	369.34 ± 24.83 A	235.34 ±16.38 aB	48.34 ± 4.41 ab
4wpv	152.00 ± 27.87 A	130.67 ±14.90 B	45.67 ± 2.34 ab
	Booster dose of vaccine		
6wpv	756.34 ±95.69 A	582.00 ± 25.17 B	51.67 ± 2.34 ab
8wpv	1760.34 ± 30.02A	1548.67 ± 25.10 aB	50.00 ± 2.89 ab
10wpv	1727.67±14.68A	1641.34 ± 25.46 aB	49.34 ± 2.96 ab
12wpv	1658.00 ±11.72A	1556.67 ±12.44 aB	49.34 ± 2.96 ab
14wpv	1423.00 ±23.52 A	1204.67±16.22 aB	49.34 ± 0.24 ab
16wpv	1125.00±41.88 A	1052.67±36.14 B	50.00 ± 1.15 ab
18wpv	999.77 ±41.74 A	915.00 ± 54.05 B	50.00 ±1.15 ab
20wpv	816.67 ±65.83 A	685.67± 13.09 aB	57.67 ± 1.20 ab
22wpv	632.00 ± 25.99 A	489.67 ± 25.76 Ab	52.64 ± 1.20 ab
24wpv	313.67 ± 25.58 A	210.71 ± 13.09 aB	49.00 ± 0.58 ab
26wpv	231.00 ± 27.43 A	119.67± 9.61 aB	49.00 ± 0.58 ab
28wpv	199.00 ± 24.99 A	96.31 ± 8.57 a	51.34 ± 1.20 ab

Group I: vaccinated with inactivated EHV-1vaccine encapsulated with chitosan nanoparticles in rtio of (1:1).

Group II: Vaccinated with inactivated EHV-1 adjuvanted with gel (commercial vaccine)

Group III: unvaccinated control.

The mean difference is significant at 0.05 level. Small letters against capital letters in the same row indicate significant difference between groups (using ANOVA test).

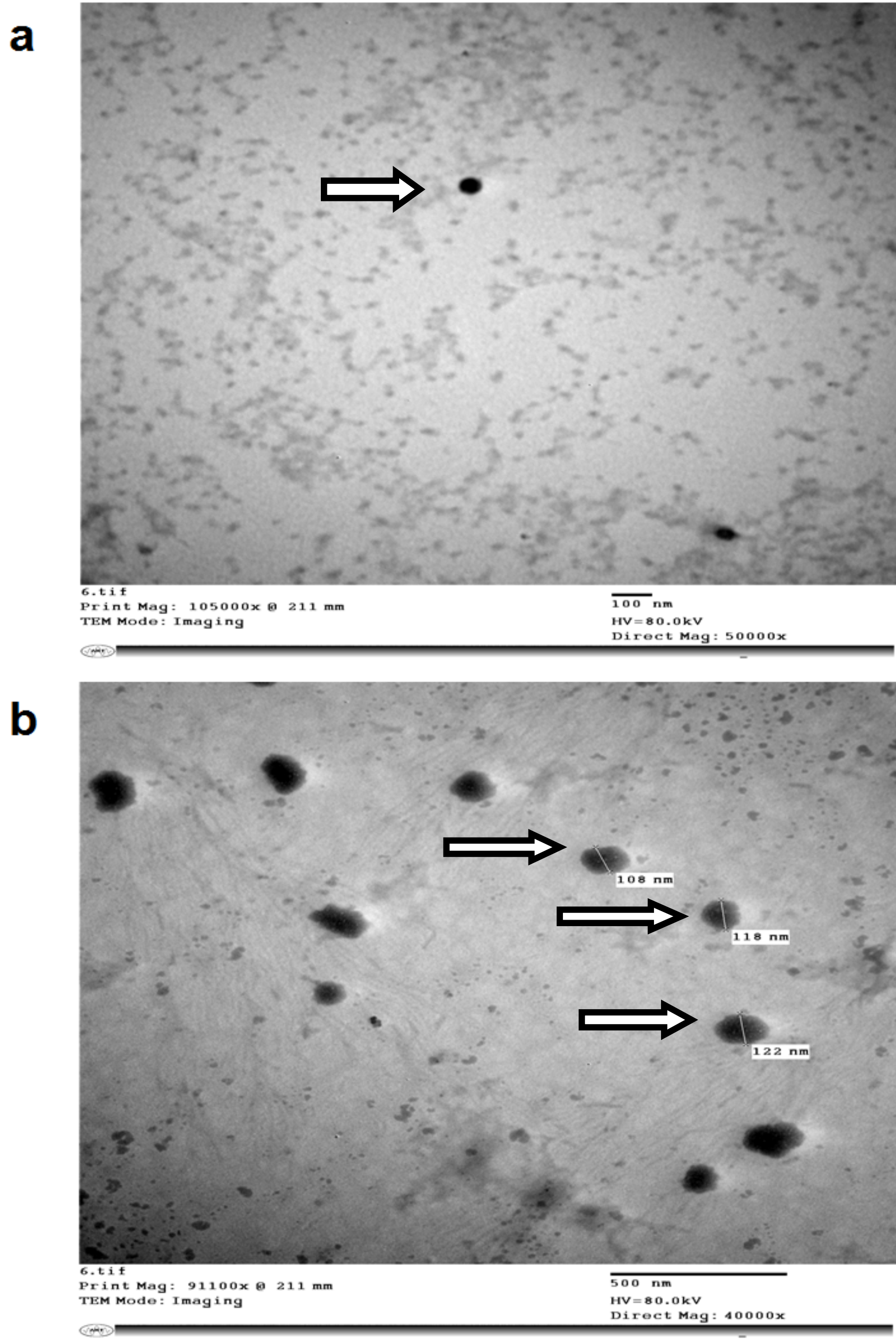
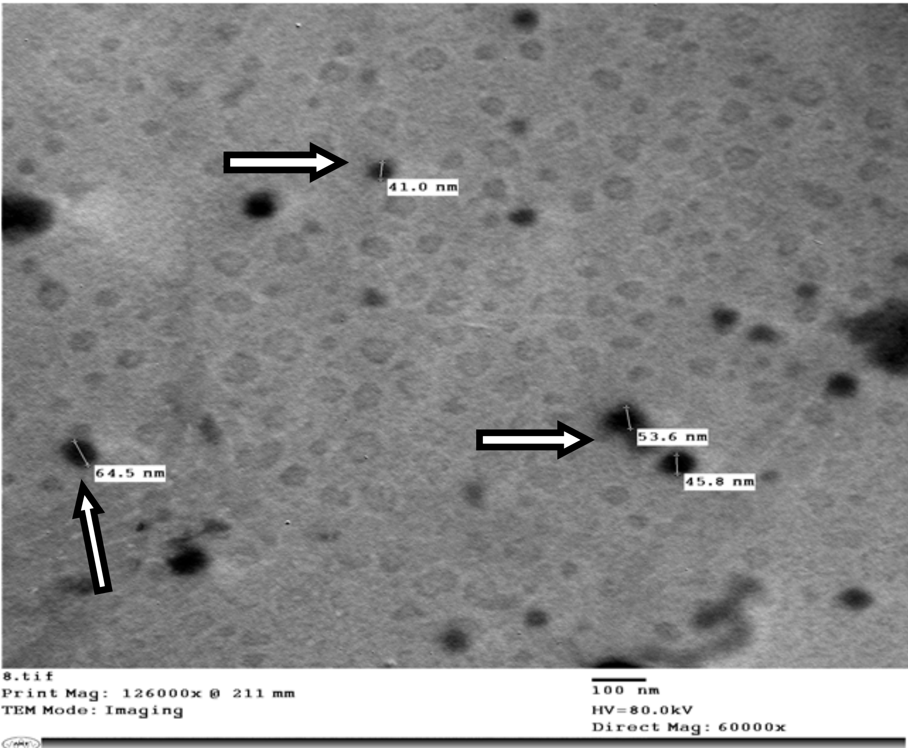


Fig. (1): TEM image of chitosan nanoparticles of (50 nm to 60 nm) (a) EHV-1 encapsulated in chitosan nanoparticles (100 nm to 125 nm) (b)



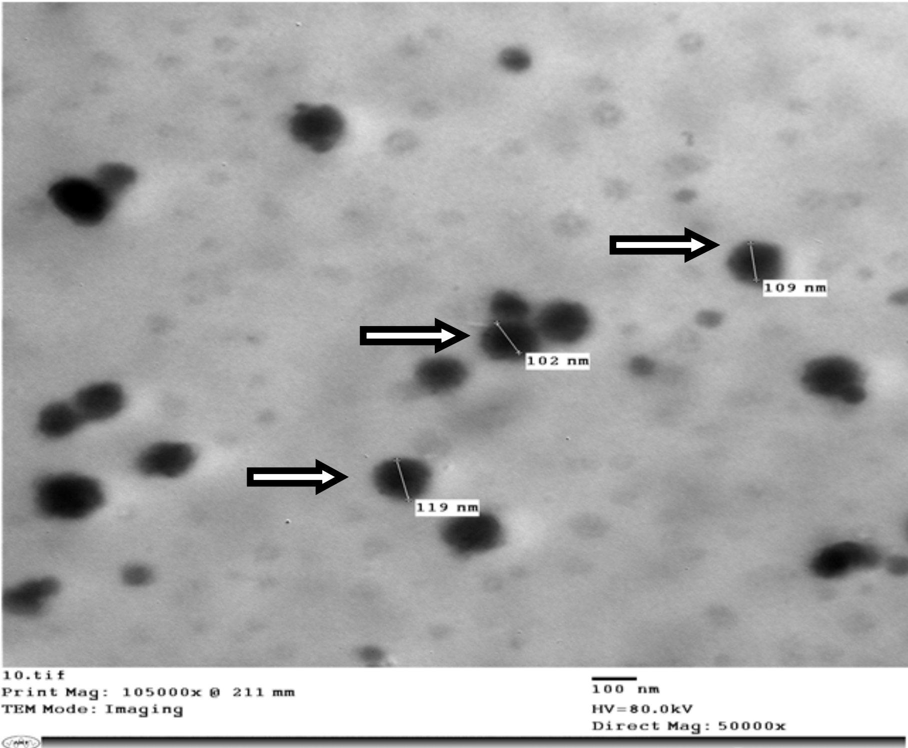


Fig (2): TEM image of calcium phosphate nanoparticles of (40 nm to 70 nm) (a) EHV-1 encapsulated nanoparticles in calcium phosphate nanoparticles (100 nm to 120 nm) (b).