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Effect of Clinoptilolite on Humoral Immunity and Biochemical Parameters in Calves Vaccinated with Foot and Mouth Disease Vaccine

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Abstract: The foot and mouth disease (FMD) vaccine is used to control the FMD in cloven-hoofed animals including calves. The most important step in vaccine preparation is the selection of a suitable adjuvant which plays a main role in the vaccine's potency. The present study is an attempt to use clinoptilolite instead of Montanide oil to enhance the efficiency of the FMD vaccine and reduce its production cost. Twelve calves were split into four groups. Three groups were vaccinated with FMD vaccine with different adjuvants i.e. 100% Montanide (ISA 206), 50% of Montanide with 1.0 µg/dose of clinoptilolite (ISA 206 + CLINP), and by replacing the 100% of Montanide by 1 µg/dose of clinoptilolite (CLINP). The fourth group was unvaccinated. Results showed that calves vaccinated with (ISA 206 + CLINP) FMD vaccine expressed the highest and longest periods of immune response until 38 weeks. While groups vaccinated with (CLINP) or (ISA 206) vaccine showed only protection up to 26 and 34 weeks respectively. Also, our results showed that clinoptilolite improved organ functions resulting from the stress of vaccines. Finally, this study recommended the use of clinoptilolite with Montanide oil as a potential adjuvant in the FMD vaccine to give high stimulation of the immune response.

1 Introduction

Foot and mouth disease (FMD) was recognized as a highly contagious viral disease affecting various species of cloven-hoofed animals (Sobhy et al 2018). The FMD virus (FMDV) is an *aphthovirus* that belongs to the *Picornaviridae* family (Salam et al 2014). FMDV can be transmitted immediately from infected to uninfected animals in the same place or indirectly spread among susceptible animals by contaminated air or through people. One of the countries that have spread the FMD virus is Egypt and it has taken many outbreaks have occurred since 1950 and onwards. The FMDV serotype (O) was the most epidemic until serotype "A" appeared in 2006. Locally, several outbreaks of FMD have been recorded among calves and buffaloes and was confirmed in mid-February 2006 that it is serotype "A" (OIE 2006). After that, at the beginning of 2012, the OIE recorded that there were cases numbers of FMD serotype SAT2 infectious in Egypt. In addition to this, endemic serotypes (A and O) still spread in the country (Lockhart et al 2012).

Vaccination is the first line on a large scale to control disease. Vaccines provide the most defensive and

cost-efficient means to prevent this infectious disease. One of the most important steps in vaccine preparation is the selection of a suitable adjuvant which plays a main role in the elevation of the vaccine efficacy. Most commercially available FMD vaccines are considered inactivated whole-virus preparations containing Montanide ISA 206 oil emulsion as an adjuvant to improve their efficacy and to elevation of immune response in vaccinated calves (Robinson and Christley 2007). The blood immune cell parameters showed a positive immunological response due to their activation by the vaccine antigens (Chukwuedo et al 2016). Some studies showed that the immunization of the inactivated FMD vaccine with Montanide 206 oil was capable of could induce 80% to 100% protection (Khalifa et al 2017). So, significant steps have been made to improve the quality of these vaccines by using other adjuvants (Brown and Bevins 2019).

Clinoptilolite is a natural clay that contains aluminum silicates that have formed from oxygen, aluminum, and silicon. SiO_4 and AlO_4 tetrahedral are the smallest units that give a specific shape to the molecule. Clinoptilolite has special and outstanding physical and chemical properties. These characteristics make them very useful in various applications, including agriculture, animal feeding, veterinary medicine, and agronomy. Newly, the application of clinoptilolite has been notarized in human medicine and pharmaceuticals (Kraljević Pavelić et al 2018). Clinoptilolite has a high affinity toward cations and plays the main role in regulating the immune system, so may prove useful as an adjuvant to the standard therapy (Yao et al 2016).

This work was designed to evaluate the replacement of the adjuvant of commercial vaccine, Montanid ISA 206 oil, with clinoptilolite by 50% or 100% on the immunological changes by determination of serum neutralization test (SNT). Also, the determination of the effect of the replacement of Montanid, with clinoptilolite as an adjuvant of the FMD vaccine on blood biochemical parameters in calves, was highlighted.

2 Materials and Methods

2.1 Animals

Ten-month-old calves "Egyptian Balady calves" (n = 12), were used in our study and tested negative for FMD serotype antibodies by serum neutralization test (SNT). The experiment was carried out in the Veterinary Serum and Vaccine Research Institute (VSVRI). The use of protocols and calves was confirmed by the Animal Care and Use Committee of VSVRI, Egypt.

2.2 FMD viruses

A locally isolated FMD virus strains (O PanAsia-2, A/Iran/05, and SAT2/EGY/2012) were provided from VSVRI, Abbassia, Egypt, and used in vaccine preparation and serum neutralization test (SNT). The viral serotypes were stored at – 70°C. The BHK-21 cell line was used to propagate the three serotype seed viruses and the harvested viruses were inactivated chemically by binary ethylene imine (BEI) according to Ismail et al (2013).

2.3 Preparation of vaccines

Three vaccines were prepared by using two types of adjuvants: Montanid ISA 206 and clinoptilolite. The antigen: adjuvant ratios were agreed according to the procedure described by Barnett et al (1996). Montanid ISA 206 is a mineral oil-based adjuvant that forms a water/oil/water emulsion or a double emulsion (continuous aqueous phase emulsion inside which droplets of oil contain a second aqueous phase) without the need for surfactant. It was obtained from Seppic, Paris, France.

Clinoptilolite is the fine powder of natural clay obtained by Micronisiertes Klinoptilolith – Hochwertigs Naturminera, Germany. Adding 1 µg/dose of clinoptilolite to FMD inactivated virus mixture was according to Mansouri et al (2013).

2.4 Safety and sterility of the prepared FMD vaccines

A safety test was conducted by OIE (2006) to confirm the absence of any abnormal local or systemic adverse reactions post-vaccination. A sterility test was conducted to confirm the freedom of the prepared vaccines from any contaminations.

2.5 Experimental design of animal groups

In our study, twelve calves were split into four groups, each group contains three calves. The first group was vaccinated intramuscularly with FMD Montanide ISA 206 oil (ISA 206), the second group was vaccinated with FMD vaccine containing 50% Montanide ISA 206 oil/dose with 1.0µg/dose of clinoptilolite (w/ ½ w oil/ 1.0 µg/dose clinoptilolite) (ISA 206+CLINP), while the Third group was vaccinated with trivalent FMD vaccine by replacing the

100% of Montanide ISA 206 oil by 1μg/dose of clinoptilolite (CLINP) and the fourth group was unvaccinated and used as control. The humoral response of calves was estimated by serum neutralization test (SNT). Evaluation of biochemical parameters involves serum urea and creatinine concentrations, serum aspartate transaminase (AST) EC 2.6.1.1, alanine transaminase (ALT) EC 2.6.1.2 activities, total protein, albumin, globulins, and albumin/globulin ratio.

2.6 Evaluation of humoral response

Serum samples were collected from the vaccinated groups and unvaccinated calves weekly for one month then every 2 weeks until 40 weeks for evaluation of antibody titers against FMD virus strains (O/pan/Asia2, A/Iran/05 and SAT2/EGY/2012) using the neutralization assay as described previously (WOfA Health 2012) and The SNT was calculated according to Reed and Muench (1938).

2.7 Biochemical analysis

Serum samples were taken at time zero, after that through 1, 2, 3, 4, 6, 8, and 10 weeks for biochemical analysis. Biochemical parameters, serum urea concentration was determined using the method of Artiss and Entwistle (1981) while creatinine concentration was measured according to (Bowers and Wong 1980). The methods for the determination of ALT and AST activities are optimized according to Henry (1964).

Serum total protein was determined using the method of Yatzidis (1987), and albumin was measured according to Doumas and Biggs (1972). The concentration of serum globulins was calculated by subtracting serum albumin concentration from total protein concentration.

Our parameters were performed by the spectrophotometric method by using commercially available test kits provided by Biomed Diagnostics Company (Germany) except the creatinine kit that was supplied by Spectrum Diagnostics Company (Germany).

2.8 Statistical analysis

The obtained data were analyzed using the Graph Pad Prism 8 program by using two-way ANOVA to conclude the statistical significance of differences among groups. The data are presented as means of individuals ± standard deviation (SD).

3 Results and Discussion

3.1 Titration of FMDV harvests after inoculation on BHK-21 cells

The FMD virus harvests were titrated in tissue culture plates using Baby Hamster Kidney (BHK-21) cells to detect the infectivity titer symbolized as $\log_{10}$ $TCID_{50}$/ml. They were $10^{8.5}$, 10^{8}, and $10^{7.6}$ respectively with FMDV serotype **(**O/pan Asia2, A/Iran 05 and SAT2/EGY/2012).

3.2 Immunological responses of FMD vaccines

FMD vaccine enhances the humoral immune response in the vaccinated calves, and there was a good engagement between antibody levels and protection against live virus challenge for the same strain of studied virus from which the vaccine was produced (Brown 1999). In general, it is recognized that a higher concentration of immunoglobulins gives more protection against FMD.

The immune response to the first vaccination depends on the antigen's dose and additive adjuvants which increase antibody production and produce a higher level of immune response (Park 2013). Therefore, the vaccines that have recently been used worldwide improve immunity and secure long-lasting antibodies by using the oil for adjuvants. On the other hand, the oil-adjuvant vaccine significantly increases humoral immunity and has superior antibody formation (Doel 1996). Humoral immunity was evaluated by SNT.

After vaccination against the FMD virus, the antibody titers of calves were evaluated using the SNT (McCullough et al 1992). **Table 1** shows the means of serum neutralizing test (SNT) in three groups post-vaccination. Calves vaccinated with inactivated FMD (ISA 206) vaccine recorded protective antibody titer at 2^{nd} WPV (1.55^{b} ± 0.09), for three serotypes (O, A, SAT2) while, animals vaccinated with inactivated FMD (ISA 206 + CLINP) vaccine recorded protective antibody titer at 1^{st}-week post-vaccination (WPV) (1.70^{c} ± 0.09) for serotype O, (1.75^{b} ± 0.09) for serotypes A and SAT2. Also, calves vaccinated with inactivated FMD (CLINP) vaccine recorded protective antibody titer at 1^{st} WPV (1.55^{cb} ± 0.09) for serotypes O and SAT2, (1.6^{b} ± 0.09) for serotype A. The protective antibody titers of the vaccinated groups were increased gradually until peaking at the 12^{th} WPV (2.97^{c} ± 0.03) for serotype O, (3.05^{c} ± 0.03) for serotypes A and SAT2 with (ISA 206) vaccine. Also (ISA 206+CLINP) vaccine reached a peak at 12^{nd} WPV (3.25^{c} ± 0.09) for serotypes A and SAT2, (3.1^{c} ± 0.03)

for serotype O. While the group vaccinated with (CLINP) vaccine reached a peak at 10th WPV ($2.9^{b}\pm$ 0.09) for serotypes A and SAT2, (2.6^{b} ± 0.09) for serotype O and failed to protect animals for a long time whereas the antibody titer decreased below by 26th week (1.55^{b} ± 0.09) for three serotypes, till the end of the experiment. The protective antibody titer slightly decreased in (ISA 206+CLINP) compared to (ISA 206) by the end of our study at the 38th and 32nd WPV respectively. This can be interpreted according to Emmer et al (2014) who found that the antigen-activated T-lymphocytes induce the humoral and cellular immune response. Also, lymphocytes can be enhanced by silicates, which are also considered as foreign bodies. They have already been conducted for different silicate materials in lab conditions and this mechanism may be understood as the immunomodulatory activity of clinoptilolite (Aikoh et al 1998). Our results show that the incorporation of the (ISA 206+CLINP) inactivated FMD vaccine stimulates the protective immune response in calves. Additionally, using clinoptilolite as an adjuvant in vaccines can enhance the humoral immune response.

Fig 1 shows the period of protection for three groups post-vaccination for FMD serotypes. Calves vaccinated with the inactivated FMD (ISA 206+CLINP) vaccine recorded higher and longer protection periods and reached 38th WPV than the other groups for three serotypes. This may be attributed to the composition of clinoptilolite as a basic material containing silicates which in particparticular stimulates the immune response (Jurki´c et al 2013).

3.3 Serum biochemical analysis

This study also shows the effect of the FMD vaccine with different adjuvants on biochemical parameters. Detection of variations in enzyme concentrations presented in the serum can spot tissue and/or cellular damage (Yun et al 2014). Urea and creatinine concentrations in the sera of mice were used to determine the renal function and show its integrity (Thumamo-Pokam et al 2018). Collected blood samples from vaccinated animals on different days after FMD vaccination until 7th DPV, recorded that the blood urea nitrogen levels, AST and ALT activities were higher than those of the control group (Cha et al 2017). Data presented in **Fig 2** reveal the urea and creatinine levels in calves vaccinated with inactivated FMD vaccine. The urea levels increased significantly after vaccination from the 2nd week until the 10th WPV compared to the control in both groups vaccinated with (ISA 206) and (ISA 206+CLINP). However, this increase in urea levels was in the normal range. Also, **Fig 2** showed a high increase in creatinine concentration at the 2nd WPV which is considered above normal level and started to decrease gradually till the 10th WPV in only (ISA 206) group compared to other groups. The calves vaccinated with inactivated FMD vaccine (ISA 206+CLINP) showed a slight increase in creatinine levels at the 2nd WPV and quickly returned to the normal range at the 3rd WPV. In this respect, clinoptilolite is a good material as an adjuvant to the standard therapy because clinoptilolite has material properties which more affinity toward ammonium which is the end-product of protein fermentation (Yao et al 2016).

Table 1. A statistically significant of the means of serum neutralizing test (SNT) in three groups at week post-vaccination for all serotypes of FMD[1]

Groups	ISA 206[2]			ISA206+CLINP[3]			CLINP[4]		
	Start	peak	end	Start	peak	end	Start	peak	end
Week Post Vaccination	2	12	34	1	12	38	1	10	26
Serotype O	1.55 ± 0.09^{b}	2.97 ± 0.03^{c}	1.55 ± 0.09^{c}	1.70 ± 0.09^{c}	3.1 ± 0.03^{c}	1.55 ± 0.09^{c}	1.55 ± 0.09^{c}	2.6 ± 0.09^{b}	1.55 ± 0.09^{b}
Week Post Vaccination	2	12	34	1	12	38	1	10	26
Serotype A	1.55 ± 0.09^{b}	3.05 ± 0.03^{c}	1.55 ± 0.09^{c}	1.75 ± 0.09^{b}	3.25 ± 0.09^{c}	1.60 ± 0.09^{c}	1.6 ± 0.09^{b}	2.9 ± 0.09^{b}	1.55 ± 0.09^{b}
Week Post Vaccination	2	12	32	1	12	38	1	10	26
Serotype SAT2	1.55 ± 0.09^{b}	3.05 ± 0.09^{c}	1.6 ± 0.09^{c}	1.75 ± 0.09^{b}	3.25 ± 0.09^{c}	1.65 ± 0.15^{c}	1.55 ± 0.09^{b}	2.9 ± 0.09^{b}	1.5 ± 0.09^{b}

1 Values with different small letters within a row are significantly different ($P<0.05$).
2 ISA 206: Calves vaccinated with inactivated FMD vaccine with Montanide ISA 206.
3 ISA 206+CLINP: calves vaccinated with inactivated FMD vaccine with 50% of Montanide ISA 206 with 1 µg/dose clinoptilolite
4 CLINP: calves vaccinated with inactivated FMD vaccine with 1 µg/dose clinoptilolite

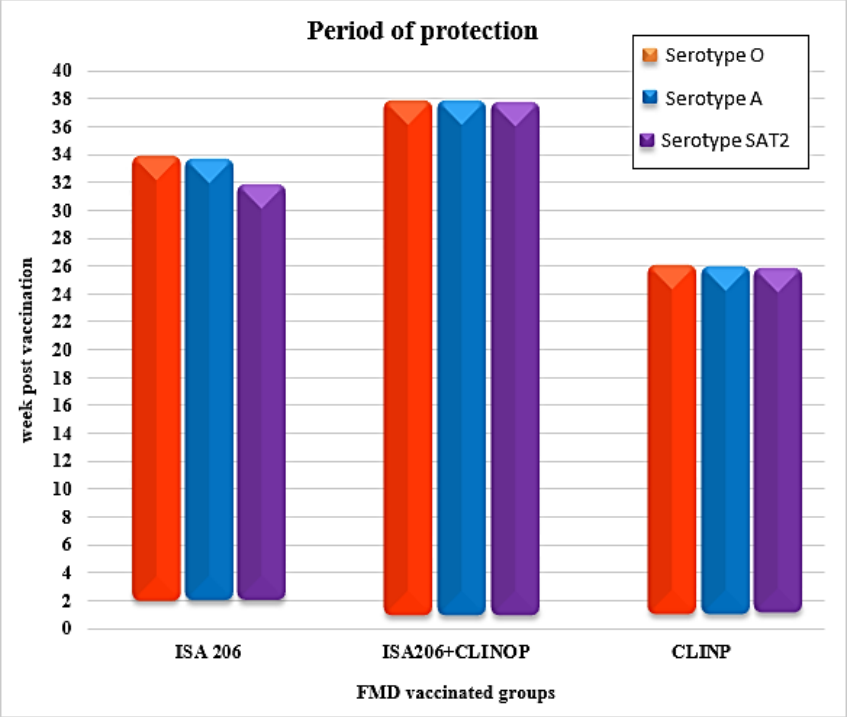

Fig 1. Period of protection in vaccinated calves with different adjuvants of inactivated FMD vaccines during 40 weeks after vaccination. ISA 206: calves vaccinated with Montanide ISA 206 inactivated FMD vaccine, ISA 206+CLINP: calves vaccinated with 50% of amount of Montanide ISA 206 + 1µg/dose clinoptilolite inactivated FMD vaccine, CLINP: calves vaccinated with 1µg/dose clinoptilolite inactivated FMD vaccine

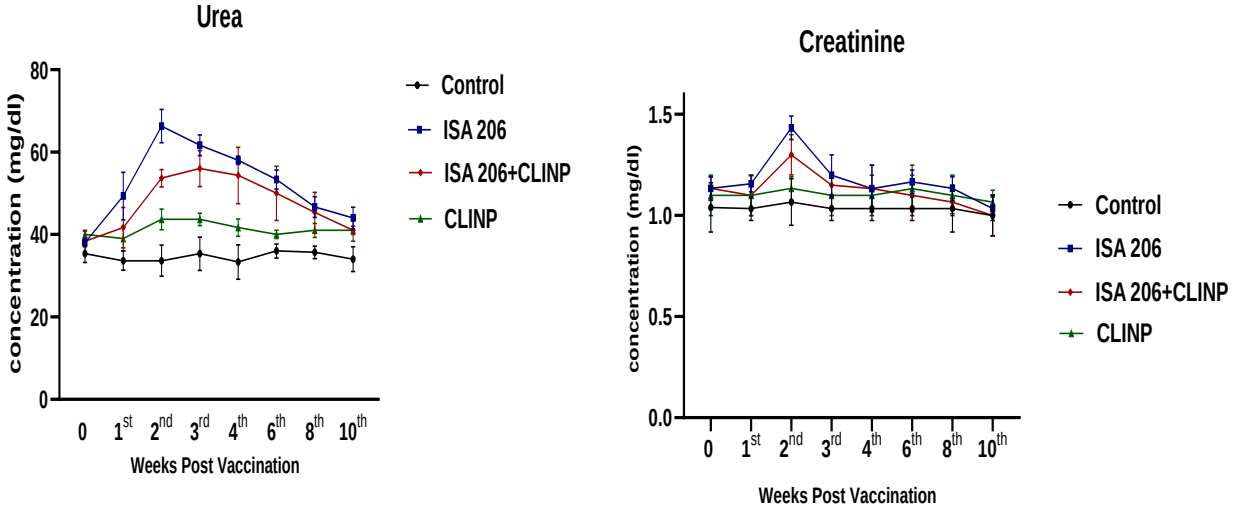

Fig 2. Urea and creatinine concentrations in vaccinated calves with different adjuvants of inactivated FMD vaccines started by time zero till 10th weeks post-vaccination. Data are shown as means ± SD. ISA 206: calves vaccinated with Montanide ISA 206 inactivated FMD vaccine, ISA 206+CLINP: calves vaccinated with 50% of Montanide ISA 206 with 1 µg/dose clinoptilolite inactivated FMD vaccine, CLINP: calves vaccinated with 1 µg/dose clinoptilolite inactivated FMD vaccine

Fig 3 shows the activities of AST and ALT enzymes in the blood serum of calves in different groups. The obtained data revealed no significant changes in ALT in all groups. However, AST significantly increased with only the inactivated FMD vaccine (ISA 206). In calves, some farm operations caused many stress conditions such as vaccination, and transportation. This stress leads to increased cortisol (stress hormone) concentrations in blood and modulation of inflammatory cytokines (Kim et al 2011).

In addition, this increase in serum AST and urea may be related to glucocorticoid rise so stress can elicit an apparent increase in serum AST and urea concentration (Shawky et al 2016). The increase of AST activity after vaccination as shown in **Fig 3** indicated that the vaccination may induce damage effects to liver cells. Nath et al (2014) observed that the calves conjugated with the FMD vaccine had greater AST activity compared to the control group suggesting that the effects of the FMD vaccine caused liver damage. This can be attributed to the oxidative stress of vaccination. This oxidative stress causes liver damage leading to an increase of released enzymes from liver cells into the blood (Swain et al 2000). Clinoptilolite showed positive effects on hepatocytes which leads to a decrease in the level of malondialdehyde, a marker of oxidative stress, in the liver tissue (Saribeyoglu et al 2011).

Furthermore, results showed in **Fig 4** Indicate the levels of total protein, albumin, globulins concentrations and A/G ratio in all calves vaccinated with inactivated FMD vaccine. The three vaccinated groups were significantly higher in total protein and globulins concentrations after vaccination from the 1st WPV until the 10th WPV than those in control. However, the group vaccinated with inactivated FMD vaccine with (ISA 206+CLINP) was the highest after vaccination**.** Conversely, no significant differences in the levels of serum albumin were observed among vaccinated groups compared with control during this period. In this respect, the significant increase in total proteins in the vaccinated groups may be due to the production of antibodies after vaccination. Our results were confirmed with those obtained by Çiftci et al (2019).

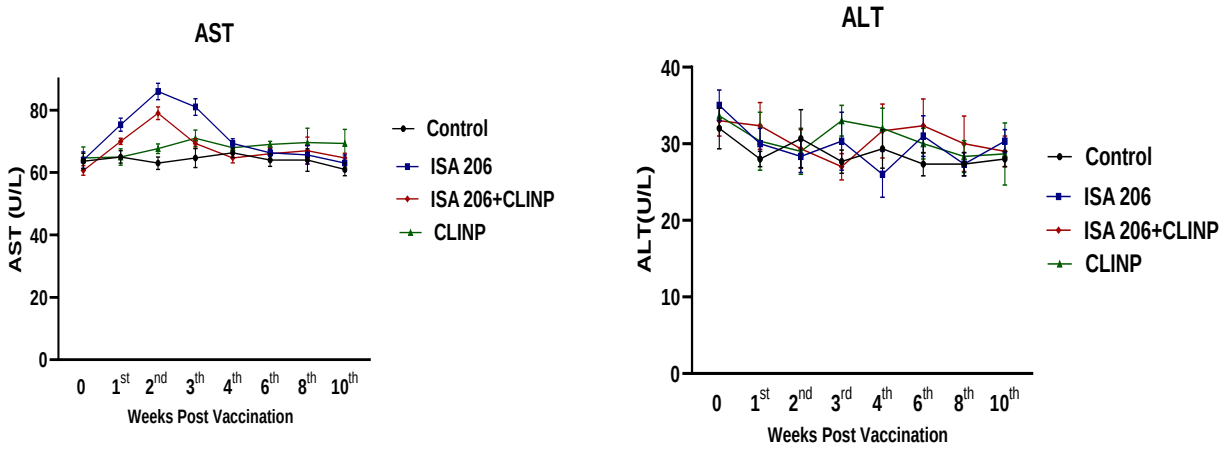

Fig 3. Liver function tests in vaccinated calves with different adjuvants of inactivated FMD vaccines before vaccination till 10th weeks post-vaccination. Data are expressed as means ± SD and the significance. ALT: alanine aminotransferase, AST: aspartate aminotransferase. ISA 206: calves vaccinated with Montanide ISA 206 inactivated FMD vaccine, ISA 206+CLINP: calves vaccinated with 50% of Montanide ISA 206 with 1µg/dose clinoptilolite inactivated FMD vaccine, CLINP: calves vaccinated with 1µg/dose clinoptilolite inactivated FMD vaccine

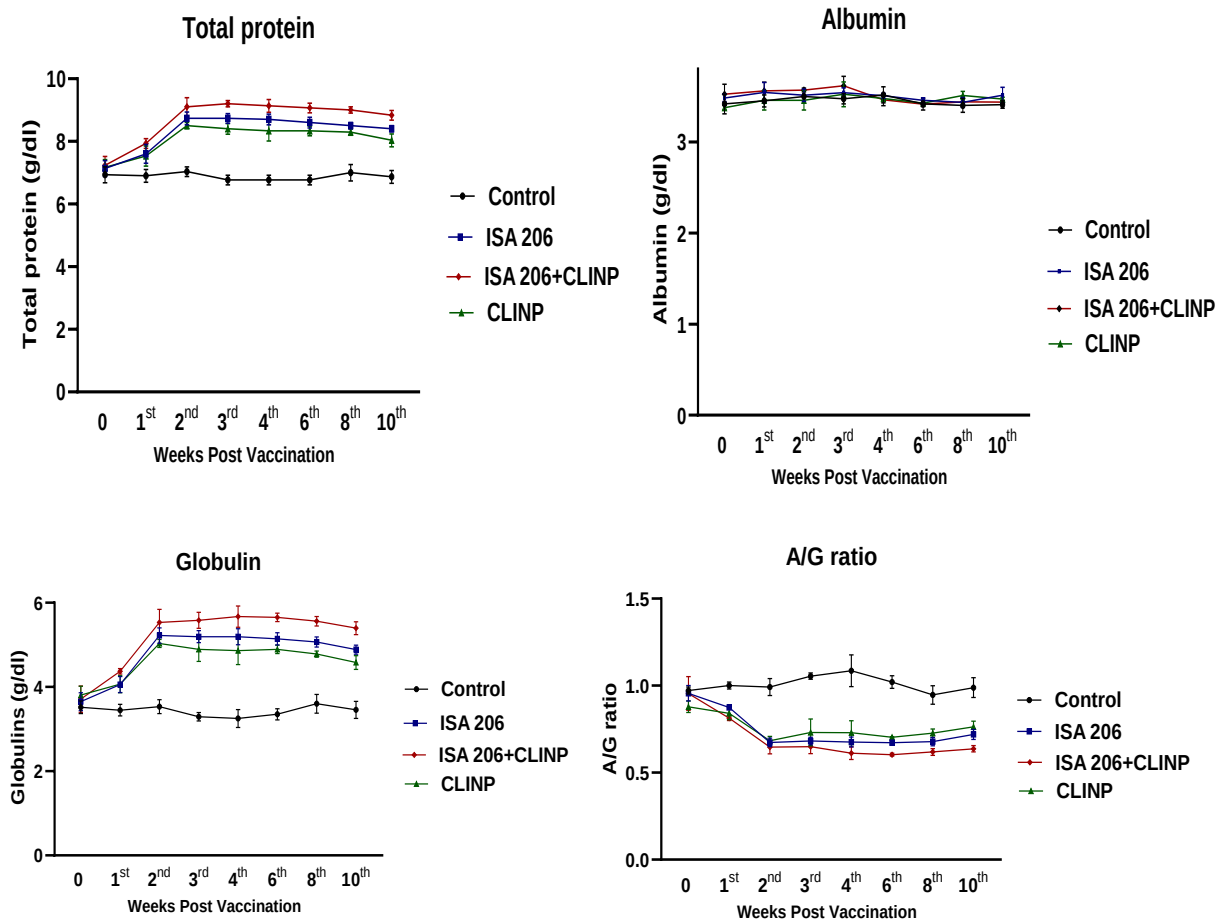

Fig 4. Total proteins, albumin, globulins, and albumin/globulin ratio (A/G) in vaccinated calves with different adjuvants of inactivated FMD vaccines before vaccination until 10th weeks post vaccination. Data are expressed as means ± SD and the significance. ISA 206: calves vaccinated with Montanide ISA 206 inactivated FMD vaccine, ISA 206+CLINP: calves vaccinated with 50% of Montanide ISA 206 with 1 µg/dose clinoptilolite inactivated FMD vaccine, CLINP: calves vaccinated with 1µg/dose clinoptilolite inactivated FMD vaccine

In the present study, serum globulin levels increased significantly while A/G ratios decreased in all vaccinated groups compared to the control. Specifically, (ISA 206+CLINP) vaccinated calves showed significantly the highest levels of globulin and the lowest ratio of A/G compared to other vaccinated groups. These results reflected that the vaccination against FMDV with clinoptilolite can stimulate the humoral immune response in vaccinated calves and increase antibody production. These results were agreed with those of Park (2013). It is generally accepted that the addition of clinoptilolite at the (ISA 206+CLINP) vaccinated group formulation induced an increase in antibody level and the concentration of serum immunoglobulins in comparison with the other vaccinated groups (Rigden et al 2003). Our results were in agreement with Koch et al (1980) who demonstrated that the enhancement effect of vaccination on globulin concentration in serum when oral or parenteral immunization has been attributed to the reaction of the immune-globulin producing system to the redaction of tissue proteins or antigens. Also, our results concerning globulin concentration were in agreement with previous studies obtained by Stojić et al (2020) who indicate that the clinoptilolite-based mineral adsorbed in the colostrum led to an increasingly significant IgG concentration in the serum of calves. Our hypothesis is that the addition of clinoptilolite into vaccine formulations as an adjuvant could generate an immune response and give more protection.

4 Conclusion

Using clinoptilolite as an adjuvant with Montanide ISA 206 oil in FMD trivalent vaccine induces long-lasting immunity more than the oil adjuvant alone or

with clinoptilolite alone and improves humoral immunity. Furthermore, it is not toxic and can improve organ functions resulting from the stress of vaccines. So, it is recommended to use the FMD-inactivated vaccine adjuvant with oil and clinoptilolite in the companying of vaccination to control FMD. We also recommended increasing the studies on the effect of clinoptilolite as an antioxidant in veterinary.

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