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A trial for preparationand assessment of montanide gel 01 adjuvanted inactivated strangles vaccine.

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This study declared a trial forthe preparation and assessment of Montanide gel 01 adjuvant inactivated Strangles vaccine. This vaccine was prepared from *Streptococcus equi subs. equi*(field isolate)that was confirmed by PCR. The prepared vaccine was assessed by using two species of animals, horse as a main host and rabbit as a laboratory animal. A total of 10 male horses were equally divided into 2 groups' first one wasvaccinated with the vaccine, the secondone was control and serum antibodies titer was measured for two groups for six months. Twenty New-Zealand rabbits were used as follow; fifteen rabbits were divided into 2 groups. The first group contains 6 rabbits were used as a vaccinated group which was vaccinated and challenged, the second group 9 rabbits were used as a control was divided into 2 groups (one contains 6 (positive control) were infected intendedly at the challenge time and another containing 3 were negative control). Another five rabbits were used in a purity of vaccine. ELISA results of horse showed high significant value in the vaccinated group than control along the study. ELISA results of rabbit also showed highly significant value in the vaccinated group than the positive control group on along the study except for 2nd and 3rd weeks post challenge. The result of histopathologicalexamination of various sections of submaxillary lymph node of negative control rabbits revealed normal lymphoid follicles While in positive control showed nuclear pyknosis, karyorrhexis or karyolysis, with the presence of empty areas and eosinophilic cellular debris in some other areas. However, the vaccinated group showed marked activation of their germinal centers with the appearance of many tangiblebodies, macrophages, and some apoptotic cells and Mild to moderate degree of lymphocytic degenerative and necrotic changes. Briefly, the prepared vaccine was given good protection.

Keywords: Strangles, *S.equi*, ELISA, MONTANIDE GEL 01, histopathological, lymph nodes.

INTRODUCTION

Strangles is an infectious respiratory bacterial disease of Equidae. It is considered one from the most 3 significant respiratory diseases of horses. (Natarajan, D. and Langohr, I. 2003) that caused by *S. equi* which is under Lancefield group C and cause Beta hemolysis on blood agar (Slater, 2007). A higher occurrence of *S. equi* was recorded in the young foals compared to the adult one. *S. equi* easily spreads from infected to susceptible horses through contaminated water. (Neamat-Allah, A. N.F,and El Damaty, H.M. 2016).Streptococcus is a genus of coccus (spherical) Gram-positive bacteria belonging to the phylum Firmicutes Ryan (Sims et al., 2004) and the order Lactobacillus (lactic acid bacteria).In

1984, many bacteria formerly considered *Streptococcus* were separated out into the genera *Enterococcus* and *Lactococcus*(Facklam, 2002).This genus had been found to be part of the salivary microbiome(Wang et al., 2016).*Streptococcus* were classified according to their hemolytic properties into alpha, beta,and gamma. Alpha-hemolytic species cause oxidization of iron in hemoglobin in red blood cells, giving it a greenish color on blood agar, beta-hemolytic species cause complete rupture of red blood cells while gamma-hemolytic species cause no hemolysis (Patterson, 1996).Beta-hemolytic streptococci were further classified according to Lancefield grouping, a serotype classification that is describing specific carbohydrates present on the bacterial cell wall (Facklam, 2002). There are 20 described serotypes were named Lancefield groups A to V (excluding I and J). The bacterium *S. equisubsp. equi* infects Equine and causes a disease called strangles (Sweeney et al., 2005).In Egypt, with the rapid expansion of equine trade, strangles infection formulates one of the most important of chronic bacterial diseases. Also, the introduction of foreign breeds created many problems concerning equine as a silent reservoir. Vaccines have historically been the most effective means to fight and eradicate infectious diseases (Grammatikos et al., 2009). There are numbers of living and killed strangles vaccines used commercially worldwide with some success. The live vaccine proved its efficacy but it could be hazardous because of residual virulence caused by insufficient attenuation, reversion to virulence and uncertain safety (Barbezange et al., 2000), so its use is prohibited in several countries. However, the inactivated vaccine had been developed and can confer good protection against strangles. The aim of this study to the preparation of inactivated vaccine which gave good protection and prevents the spread of Strangles.

MATERIALS AND METHODS

Experimental design:

Aten male horses about 10 – 13 months of age were equally divided into two groups one vaccinated with 1ml I/M of prepared vaccine as first and second dose with 21 days' interval and another were used as negative control. Blood samples were collected from ten horses every week after first and second vaccination dose until 2nd month then every month until the 6th month. The samples measured for antibodies titer by ELISA kit for an equine blood sample (IDvet innovative diagnostic-France)**.**Also, twenty laboratory male rabbits of 1200 – 1500 gm body weight were used in this study. Five used in the purity of prepared vaccine and fifteen rabbits were divided into two groups. First one was vaccinated group (contained 6 rabbits) and the Second one was a control group (contained 9 rabbits). Control group was divided into two groups, one was positive control (contain 6 rabbits) and another was negative control (contain 3 rabbits). The vaccinated group was injected with prepared vaccine I/M with 1 ml contained approximately 3×10^8 CFU as first and second doses with 21 days' intervals then challenged with *S. equi*after 21 days from second vaccination dose, while the positive control group was artificially infected with *S. equi*at challenge time. Blood samples were collected from three rabbits from vaccinated and positive control groups every week after first and second vaccination dose until the 10th week. These samples measured for antibodies titer by ELISA according to Briggs and Skeels (1984).Another three rabbits from vaccinated and positive control with three rabbits of the negative control group wereused in the histopathological examination of mandibular and submaxillary lymph node tissues for *S. equi*by preserved in 10% buffered neutral formalin. Formalin-fixed lymph nodes specimens were routinely dehydrated by graded series of alcohol, cleared in xylol and finally embedded in paraffin. Paraffin blocks were serially sectioned at 4-5µm thickness and stained with H&E (Bancroft and Gamble, 2008).

Bacterial strain:

*Streptococcus equi subspecies equi*were used in this study. This strain was field isolate and identified confirmed with PCR using primer has sequence ASW73 forward primer (5′-CAGAAAACTAAGTGCCGGTG-3′) and, ASW74 reverse primer(5′-ATTCGGTAAGAGCTTGACGC-3′) (Metabion - Germany).

Vaccine preparation (Formalin killed vaccine):

According to (Charles et al., 1994)a culture of field*S. equi* strain grown on Edward media (Oxoid) was sown into thioglycolatebroth (Oxoid) and incubated at 37˚C for 24 - 48 hrs. The concentration of the organism was adjusted to give the strength of 3×10^8 CFU/ml and 0.2% formalin 37% (Sigma) was added. After thorough shaking, the vaccine was left at incubator at 37˚C for 24 hrs. to allow formalin to kill all organisms.

The vaccine was then placed in the refrigerator 2 days at 4˚C for stopping formalin action,then 10% Montanidegel (Seppic) and 0.05 - 0.1g/liter thiomersal (Sigma) were added. The obtained vaccine was tested for sterility by inoculation of 0.5 ml of the vaccine in brain heart infusion broth and incubated at 37˚C for 24-48 hrs. It showed no growth of *S. equi*. Also, the vaccine was tested for safety by injection of 0.5 ml I/M in five rabbits. It showed no lesion after 14 days.

RESULTSAND DISCUSSION

ELISA test appears to be a sensitive test and variability was low as reflected by the narrow standard deviation (Heath et al., 1991). The result of measurement of horse sera antibodies titer as shown in (figure1) declared that antibodies titers of the vaccinated group were higher than antibodies titers of the control group which had no significant changes in their titers all over the study.Fahmyet al., (2010) used indirect ELISA to detect antibodies against strangles. (Hobo *et al.,* 2012) found that the horses infected experimentally with *S. equi* could be diagnosed as strangles-positive by approximately seven days after they had been infected, the period after which the clinical signs appear by using indirect ELISA. The rates of protection found in the herds studied could be related with the low immunogenicity of the commercial vaccines and with the low antigenic relationships among vaccinal and field strains (Moraes et al., 2009). Also, the protection induced by inactivated vaccines, using subunits or whole cells as antigens, is generally poor and does not protect against a challenge with pathogenic strains (Jaccobs et al., 2000). In this study, two groups of rabbits which were observed after challenge. The results of the rabbits' sera which tested by ELISA for detection antibodies titeras shown in (figure2) declared that antibodies titers of the vaccinated group were higher significant than antibodies titers of positive control group all over the study except 2nd and 3rd weeks after challenge. These differences might be due to artificial infection which increased the antibodies titer in the control group but decreased it in the vaccinated group due to neutralization of some antibodies.Woolcock (1975) suggested that immunity in horses against *S. equi* infections is chiefly humoral and a possible relationship exists between antibody levels and degree of protection. Also, Srivastava and Barnum (1981) demonstrated that most of the foals, which were immune to strangle, either as a result of vaccination or natural strangles, possessed anti-M-protein PHA titers ranging from 1/400 to 1/6400.Woolcock (1975) compared the immune response in horses during 2 strangles outbreaks with the response following vaccination. Recovered animals showed declining antibody levels 9 weeks after infection and the evidence does not support the longevity of the immune response after natural infection. Reaction to vaccination was observed in those animals with high initial antibody titer. Azevedoet al., (2006) demonstrated that the encapsulation of *S. equi* antigens in Poly-lacto-glycolic acid (PLGA) microspheres, as well as their administration by the nasal and intramuscular routes, leads to an enhancement of specific immune response, causing full protection against the virulent strain. The result of histopathological examination of various sections of lymph node of negative control rabbits revealed normal lymphoid follicles and inter-follicular spaces (Figure 3a). While examination of various sections of the submaxillary lymph node of *Streptococcus equi* injected rabbits (control positive) showed severe lymphocytic depletion at which the lymphoid follicles showed degeneration and necrosis of the lymphocytes that appeared as nuclear pyknosis, karyorrhexis or karyolysis, with presence of empty areas and eosinophilic cellular debris in some other areas (Figure 3b). The later necrosis was frequently accompanied by inflammatory cells including neutrophils and phagocytic macrophages with intracytoplasmic cellular debris. The reticular cells and mash underling that necrotic lymphocytes were clearly seen (Figure 3c). Neutrophils infiltration was observed in most sections among the necrotic lymphocytes and widely seen inside the lumen of the blood vessels (Figure3d). However, examination of the submaxillary lymph nodes of vaccinated rabbits that followed by challenge with *Streptococcus equi* showed marked activation of their germinal centers with the appearance of many tangible body macrophages and some apoptotic cells and bodies (Figure 3e). Those macrophages appeared engulfed an antigen for its processing. Mild to moderate degree of lymphocytic degenerative and necrotic changes were observed along with neutrophilic infiltration inside the vascular lumen (Figure 3f). The palatine tonsil and the mandibular lymph nodes were enlarged on clinical examination. Timoney et al., (2008) observed *S. equi* in small numbers in all tonsil tissues of yearling Horses 3 h after inoculation of biotinylated organisms.

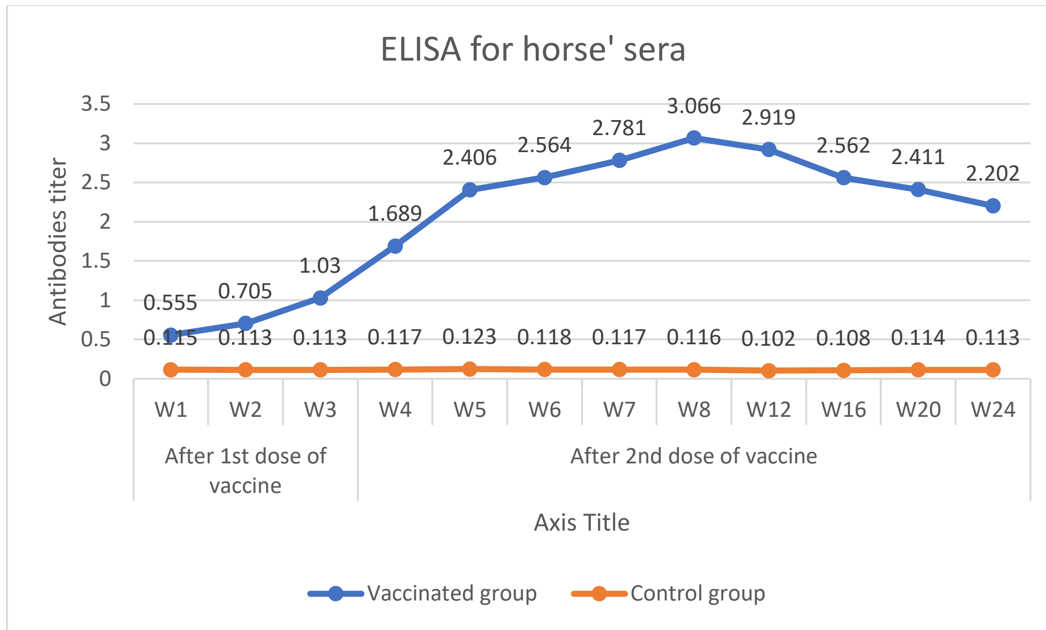

Figure (1) Antibody titers of horses vaccinated with adjuvanted Strangle Montanide Gel 01 vaccine and control group measured by enzyme-linked immunosorbent assay (ELISA).

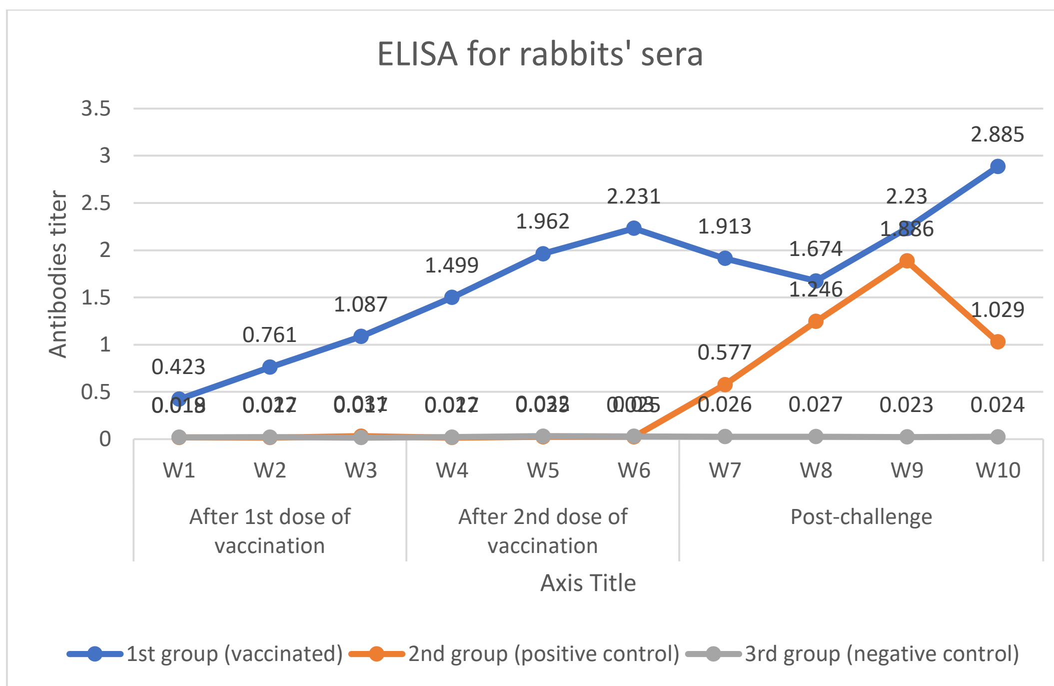

Figure (2) Antibody titers of rabbits vaccinated with adjuvanted Strangle Montanide Gel 01 vaccine and control group (positive and negative) measured by enzyme-linked immunosorbent assay (ELISA).

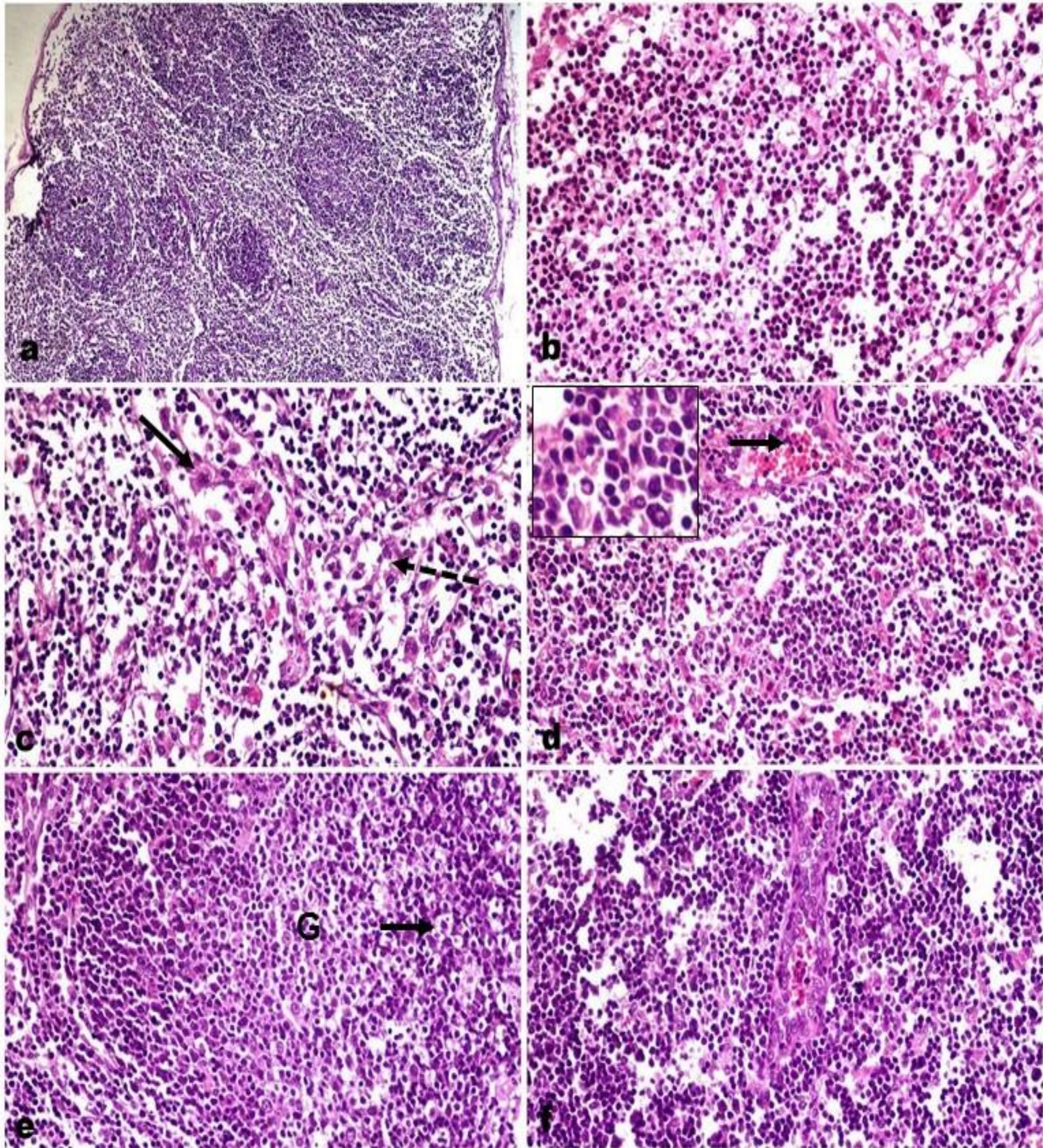

Figure 3:(a)Submaxillary lymph node of control rabbit showing normal lymphoid follicles and inter-follicular spaces, (b-d)Submaxillary lymph node of *Streptococcusequi* injected rabbit showing; (b) lymphocytic depletion, degeneration and necrosis of the lymphocytes (c)reticular cells (arrow) and mash (dashed arrow) underling that necrotic lymphocytes, (d) neutrophils infiltration among the necrotic lymphocytes and inside the lumen. (e and f)submaxillary lymph nodes of vaccinated rabbits followed by challenge with *Streptococcusequi* showing (e) activation of germinal centers (G), tingible body macrophages (arrow) and some apoptotic cells and bodies, (f) Mild to moderate degree of lymphocytic degenerative and necrotic changes. (H&E, X400).

In Weanlings, the organism was visible in small numbers in the nasopharyngeal/tubal tonsils but was rare in sections of the oropharyngeal tonsils. Fluorescent cocci were visible in two's or three's in the crypts and within the crypt epithelium. Sonmez K. and Gurel A. (2013) found the histopathological findings in lymphoid tissue samples from archived cases and from the dead foal were similar. The most remarkable changes observed in the spleen were the depletion of germinal centers. Reticular cells filled the area of missing lymphocytes and there was hyalinization in some germinal centers. In some sections, there were remarkable karyorrhexis and karyolysis in lymphoid cells.

Statistical analysis: The obtained results were subjected to Statistical analysis as all the parameters were analyzed by Independent T-testfor comparison between groups using a complete randomized design (Snedecor and Cochran, 1989). Differences with a P value below0.05were considered statistically significant. Statistically significant using SPSS (IBM Co.version 20).

CONCLUSION

In a conclusion, the prepared adjuvant in activated vaccine from field strain when given I/M has great efficacy to prevent vaccinated animal from infection and gave good protection for six months.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

AMA prepared the vaccines, vaccination of equine, challenge test, collection of blood samples for ELISA and performed ELISA. AAA designed experiments and reviewed the manuscript. EME cultivated and harvested *S. equi*strain. SSA performed Histopathological examination for samples. RA wrote the manuscript. All authors read and approved the final version.

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