Efficacy of different pneumonic pasteurellosis vaccines formula for cattle in Egypt

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Through the present work, three formula of inactivated Pneumonic pasteurellosis vaccine were prepared using P. multocida (capsular A) and M. haemolytica type A1 as antigens and Montanide™ 01Gel, Montanide (ISA206) and white mineral oil as adjuvants. All of these formulas were found to be safe in mice and calves inducing no abnormal post vaccine local or general reactions. Also all of these preparations were found to be potent revealing high protective levels of specific P. multocida and M. haemolytica antibodies as measured by IHT and ELISA. Although Montanide™ 01Gel showed the highest levels of such antibodies (3.2±0.173 for both P. multocida and M. haemolytica by IHT and 1.731 for P. multocida and 1.918 for M. haemolytica by ELISA) followed by Montanide (ISA206)(3.1±0.173 for both P. multocida and M. haemolytica by IHT and 1.685 for P. multocida and 1.696 for M. haemolytica by ELISA) while the mineral oil induced the lowest titers (2.9±0.32 for P. multocida and 3±0.3 for M. haemolytica by IHT and 1.459 for P. multocida and 1.541 for M. haemolytica by ELISA). In addition viable count in mice vaccinated with Montanide™ 01Gel showed the lowest bacterial counts followed by Montanide (ISA206) and then Mineral oil.

Keywords: P. multocida - M. haemolytica , Montanide (ISA206)- Montanide™ 01Gel.

INTRODUCTION

Pneumonic pasteurellosis is one of the most economically imperative infectious diseases of cattle with a widespread prevalence throughout the continents including Egypt (Kaud et al., 2010) and (Saed et al., 2015). The disease is described as an acute febrile course with severe fibrinous or fibrino-purulent bronchopneumonia, fibrinous pleurisy and septicemia. Infected animals may die within a few days of the onset of clinical signs, but those which survive the acute attack may become chronically infected (Lopez, 2001). The disease, in its typical clinical form, is highly infectious, often fatal and with very serious economic impact in animal industry because of high mortality, treatment cost and reduced weight gain (kabeta et al., 2015). Pasteurella is a major cause of pneumonia when combined with stress and with and without viral agent. M. haemolytica, which was formerly known as P. haemolytica, is the main causative agent of the disease (Quinn et al., 2002) and P. multocida is involved in lesion of Pneumonic pasteurellosis (Brennan et al.1997). Vaccine application is the most believed method for the control of the diseases, by gaining a high level of antibodies, rather than using other eradication or medication strategies. Vaccine efficacy relies on several variables like the nature and amount of antigen administered and the presence of adjuvants to develop immunogenicity (Stone et al., 1978). Vaccines which contain M. haemolytica and P. multocida antigens have been
available for many years but there is no definite evidence that they stimulate a satisfactory immunity (Yates et al., 1983). Oil emulsion adjuvants are particularly suitable for vaccines that will be required to give long term protection. The antigens being enclosed in the water droplets in the continuous phase of the oil is released slowly and delivers a long term stimulation of the immune system (Gupta et al., 1993). This work aims to evaluate the efficacy of pneumonic pasteurellosis vaccine formula using three different oils as adjuvants in cattle. These oils include Montanide (ISA 206), Mineral oil and Montanide™ 01 gel. The Montanide™ class of adjuvants well-established brand of vaccine adjuvants, which are previously accepted in Europe and involved in several registered commercial veterinary vaccines for food animals including cattle, fish, and poultry. These Montanide™ adjuvants have been shown to develop disease protection when combined with diverse types of antigens (Xiangdong et al., 2013).

MATERIALS AND METHODS

Locally isolated strains:
Virulent strains of P. multocida type A and M. haemolytica type A1 were originally isolated through this present work from cases of bovine pneumonia and identified by standard biochemical, serological tests and confirmed by polymerase chain reaction (PCR) using specific primers to identify virulence genes (data not published). These strains were used for preparation of Montanide™ 01Gel, Montanide (ISA 206) and Mineral oil adjuvanated vaccines and for challenge tests.

Experimental Animals

Cattle:
This study was conducted on twelve local breed Egyptian cattle, 6-8 month of age delivered by (Animal Production Research Institute) Kafr-Elshiekh government-Sakha. These animals were apparently healthy, free from parasites and were confirmed free from P. multocida, M. haemolytica and P. trehalosi antibodies through application of IHT and ELISA tests. They were divided into four groups (the first three groups were subjected to vaccination with the prepared vaccines while the last group was kept as non-vaccinated group).

Mice
Weaned swiss white mice of about 18-20 g body weights were used for evaluation of the efficiency of the prepared vaccines. These mice were obtained from the Laboratory Animals Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

Preparation of combined inactivated bivalent pneumonic pasteurellosis vaccines:
Fresh strains from P. multocida type A and M. haemolytica type A1 were inoculated into Bain and Jones media and incubated at 37°C for 24 hrs according to (Bain and Jones, 1958). The previously prepared cultures were inactivated by the addition of formalin as 37% solution to give a final concentration 1%. The inactivated cultures were divided into three portions to be adjuvanted as follow:

Montanide™ (01) gel:
It was mixed with culture in 20% using magnetic stirrer at approximately 300 rpm for 30 minutes (Seppic, 2008).

Montanide (ISA 206):
It was agitated gently in the beaker and the aqueous part was added in the ratio 1:1 at a moderate speed at 31°C for 10 minutes (Reddy et al. 1995).

Mineral oil:
According to (Stone et al., 1978), the prepared inactivated culture (aqueous phase) was emulsified in mineral oil at the ratio of 1:2 an adjuvant and sorbitan Monooilet (span80) and Polyxyethene sorbitan (tween 80) were used as oil phase and aqueous phase emulsifier respectively.

Quality control tests of the prepared vaccines formula:
Sterility:
The vaccines were examined for confirmation that the vaccines must be free from any bacterial and fungal contamination. Vaccines were cultured on nutrient agar, thioglycolate broth and Sabouraud’s dextrose agar and incubated at 37°C for 48-72 hours. Also, inoculation was made on Mycoplasma broth which was followed by cultivation on Mycoplasma agar and incubated at 37°C for 14 days on 5% CO2. The pure vaccines showed no growth on these media.

Safety test:
This test was carried out by inoculation of Swiss white mice with 0.2 ml of polyvalent
inactivated oil adjuvant vaccine of Pasteurella.

**Vaccine Potency in calves:**

Calves were divided into four groups (3 calves each). Groups (1,2and3) were inoculated s/c by two doses 2ml of each of the three prepared vaccines separately at 4 weeks interval, the group four was kept as unvaccinated control. Serum samples were collected at 2 weeks regular intervals for sero conversion through application of the following tests:

**Serological tests**

*(indirect) haemagglutination test :*

It was carried out for detection of Pasteurella antibodies according to (Carter and Rappy, 1962).

**Indirect Enzyme Linked Immunosorbert Assay (ELISA):**

It was carried out according to (Voller et al.1976) using anti bovine IgG (Sigma).

**Mouse-vaccination challenge inoculation system with local isolate P.multocida capsular A:**

Potency tests were done essentially as described by (Ose and Muenster, 1968).Six groups of 10 mice were used in all experiments. Each animal received two injections of 0.2 ml S/c at an interval of 3 weeks, and then challenged 10 days after the second injection. For challenge, *P.multocida* serotype A was grown on blood tryptose agar at 37°C overnight. A suspension was prepared in 0.85 % NaCl. Groups of ten experimental mice were challenged by intraperitoneal injection of 0.2 ml of 10^-1 to 10^-9 dilutions of the suspension. Groups of control mice were similarly challenged with 10^-1 to 10^-9 dilutions of the same suspension. Deaths were recorded daily for 3 days and the LD_{50} was calculated by the method of (Reed and Muench, 1938) as showed in Table (3). The potency of a vaccine was expressed in terms of the logs protection obtained as compared with the controls (Evans and wells, 1979).

**Viable counts in liver of control and vaccinated mice and challenged with locally isolates of *M.haemolytica* serotype A1:**

Each of ten mice was each given two doses of 0.1 ml *P.haemolytica* A1 capsule vaccine S/C with an interval of two weeks between the two inoculations. Two weeks after the second inoculation, these mice and a group of 10 un-inoculated controls were challenged I/P with 100LD_{50} *P.haemolytica* in heparin, directly following challenge and 6 hr later, five mice from each group were killed and viable counts were achieved on liver suspension (Evans and wells, 1979) as showed in Table (4).

**RESULTS AND DISCUSSION**

Choosing the adjuvants is one of the dynamic for the success of field vaccine. Current research efforts directed to improve protective immune response to pneumonic pasteurellosis infection have concentrated on utilize powerful vaccine adjuvants to help reducing lung lesions in cattle and clinical symptoms (Parker et al., 2009) and (Ibrahim et al., 2015).

Numerous types of vaccine adjuvants have been examined for their ability to potentiate the immune response to pneumonic pasteurellosis vaccine (Shah et al., 2001) and ( Munee et al., 2005). The aim of this experiment was to study the safety of several vaccine preparations contained inactivated locally isolated *P.multocida* serotype Capsular A and *M.haemolytica* serotype A1 antigens with dissimilar adjuvants and their efficacy in calves in comparison with classical produced local vaccine. Vaccine efficacy was assessed by measuring serum antibody titers in vaccinated calves using IHT and ELISA test and assessed in mice using mouse vaccination challenge system. Concerning the safety of the prepared vaccines, it was found that calves vaccinated even with double doses of prepared vaccines didn’t show adverse reactions or clinical manifestation. All contact controls remain sere-negative confirming the vaccines safety. These results agreed with those obtained by (Koves et al., 1982). The results revealed that calves vaccinated with different adjuvanated vaccines (Montanide™ 01 gel, Montanide ISA 206 and mineral oil vaccine) exhibited strong immune response. The level of antibody titers were measured by Indirect haemagglutination test for *P.multocida* serotype A as showed in Table (1) and Fig(1) started to rise after vaccination to reach its protective levels at the 4th week (2.3±0.173, 2.2±0.173 and 2.1±0) for (Montanide™ 01 gel, Montanide ISA 206 and mineral oil vaccine) respectively and increased gradually to its peak at the 16th week post vaccination (3.2±0.173.3.1±0.173 and 2.9±0.32) for (Montanide™ 01 gel, Montanide ISA 206 and mineral oil vaccine) respectively.
Table (1): Geometric mean passive HA titers of *P. multocida* serotype A and *M. haemolytica* (serotype A1)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean passive haemagglutination titers (log2/ml)</th>
<th>2 WPV**</th>
<th>4 WPV</th>
<th>6 WPV</th>
<th>8 WPV</th>
<th>12 WPV</th>
<th>16 WPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>P.V</strong></td>
<td>P.M</td>
<td>M.H</td>
<td>P.M</td>
<td>M.H</td>
<td>P.M</td>
<td>M.H</td>
</tr>
<tr>
<td></td>
<td>*<strong>P.M</strong>**M.H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>0.6±0</td>
<td>(0.7±0.173)</td>
<td>(1.7±0.17)</td>
<td>(2.3±0.17)</td>
<td>(2.6±0.173)</td>
<td>(2.9±0.173)</td>
<td>(3.1±0.173)</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.6±0</td>
<td>(1.4±0.173)</td>
<td>(1.6±0.173)</td>
<td>(2.2±0.173)</td>
<td>(2.5±0.173)</td>
<td>(2.8±0.173)</td>
<td>(2.7±0.3)</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.7±0.173</td>
<td>(1.4±0.173)</td>
<td>(1.3±0)</td>
<td>(2.1±0)</td>
<td>(1.9±0.346)</td>
<td>(2.3±0.173)</td>
<td>(2.5±0.173)</td>
</tr>
<tr>
<td>Control</td>
<td>0.7±0.173</td>
<td>(0.9±0.3)</td>
<td>(0.6±0)</td>
<td>(0.9±0.3)</td>
<td>(0.6±0)</td>
<td>(0.6±0)</td>
<td>(0.7±0.173)</td>
</tr>
</tbody>
</table>

Mean ±SD significant at P<0.05  *PV: prevaccination  **WP: week post vaccination  ***P.M: *P. multocida*  ****M.H: *M. haemolytica*

1st Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Montanide Tm 01 gel
2nd Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Montanide (ISA 206)
Also antibody titers were measured by IHT for *M. haemolytica* serotype A1 as showed in Table (1) started to rise after vaccination to reach its protective levels at the 4th week (1.8±0.463, 1.9±0.173 and 1.9±0.346) for (Montanide™ 01 gel, Montanide ISA 206 and mineral oil vaccine) respectively and increased gradually to its peak at the 16th week post vaccination (3.2±0.173, 3.1±0.316 and 3±0.3) for (Montanide™ 01 gel, Montanide ISA 206 and mineral oil vaccine) respectively. These findings were in agreement with those obtained by (Schimmel et al., 1988) and (Venkatesh et al., 1991) who found that the inactivated Pasteurella vaccines induce a high degree of immunity in vaccinated cattle.

Moreover, confirmation of humeral immune response was assessed by ELISA as showed in Table (2) it was observed that ELISA test considered accurate and sensitive test to evaluate the immune response status in animal sera due to in ELISA, only IgG titers can be determined. On the other hand, IHA and agglutination tests will not prove to be as reliable as ELISA due to these tests measure the overall IgG and IgM response (Shaveta et al., 2010) also (Al-Tarazi, 2002) found that the technique of ELISA was further sensitive 16 times than IHT in measuring antibody titers against *M. haemolytica* in sera of vaccinated SPF lambs.

The above mentioned results revealed that the classical vaccine based on span/tween 80 induced a lower level of antibody titers than those obtained with the two other prepared vaccines due to high viscosity and poor injectability in contrast of the Montanide™ 01 gel and Montanide ISA 206 developed emulsion of lower viscosity , high stability and high injectability avoiding commonly seen side effects that are associated with other mineral oil emulsion, these findings were parallel to those stated by (Hafez, 2011) so GMT and ELISA titers remained substantially higher than group vaccinated with span/tween 80 vaccine .

In addition Montanide™ adjuvants showed that it enhanced the cell mediated immune response as indicated by phagocytic activity, gamma interferon and interleukin 6 responses and induced protection against homologous challenge infection (El nagger et al., 2017) and (Nasr et al., 2016).

The use of Montanide gel as adjuvant induced enough early immune response in vaccinated cattle (Dupuis et al., 2008) and showed prolongation of antibody secretion and gives an intense immune response (El Sayed et al., 2011). Furthermore it has a mode of action as depot adjuvant, when using , was simply delay the elimination of antigens thus permit immune response to last longer in the form of sustained release of antigen, so the immune response being antigen driven respond to the presence of antigen (Tizard, 2009).

A foot and mouth disease vaccine formulated with ISA206 induced protection against the disease and elicited the strongest antibody response (Corri et al., 2016) and was stated to be responsible for increasing the immune response earlier as matched to the other oil based vaccines as termed by (Jang et al., 2010).

The potency test results of prepared vaccines are presented in table (3) using mouse vaccination protection test and challenged with homologous and heterologous locally isolates of *P. multocida* serotype A. A high level of protection was demonstrated in mice vaccinated with prepared vaccines and challenged with heterologous strain .This test based on the ability of prepared vaccine to induce protection --not simply to produce sero-conversion , It seems that *P. multocida* serotype A isolated from lung lesions not antigenically related indicating the significance of strain selection in the pasteurella vaccine preparation, these findings were assured by (Nazir et al., 2017) who stated that optimal formulation of inactivated vaccines need to appropriate antigen to match the field challenge infection. The mouse model of *M. haemolytica* infection and its use in assessment of efficacy of prepared vaccines was primarily described by (Evans and welles, 1979). In this experiment Mice were inoculated I/P with *M. haemolytica* incorporated with heparin, killed at various intervals after that viable count of bacteria was performed in liver suspension, *M. haemolytica* grow in exponential rate in liver of control mice .Mice vaccinated with *M. haemolytica* can be protected well against homologous challenge so bacterial count decreased rapidly in the liver as showed in Table (4 ).(Gilmour et al., 1979) demonstrated the correlation between the protection affected by vaccination of mice and the protection which was given to SPF lambs after challenge. In the present study comparison between these studied formula as showed in table (4) revealed that Montanide™ 01 gel and Montanide ISA 206 showed the best acceptable results as the least viable bacterial count respectively were noted in liver suspension of vaccinated challenged mice.
Table (2): *P. multocida* serotype “A” and *M. haemolytica* type “A1” ELISA antibody titres

<table>
<thead>
<tr>
<th>Group</th>
<th><em>PV</em></th>
<th>ELISA mean absorbance values/Weeks post vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 WPV**</td>
</tr>
<tr>
<td></td>
<td>***</td>
<td><strong>P.M</strong></td>
</tr>
<tr>
<td>Group1</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td>Group2</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Group3</td>
<td>0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>Control</td>
<td>0.05</td>
<td>0.08</td>
</tr>
</tbody>
</table>

1° Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Montanide™ 01 gel
2° Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Montanide (ISA 206)
3° Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Mineral oil.
Control group: Un vaccinated group  *PV: Pre-vaccination  **WPV: weeks post vaccination  ***P.M: *P. multocida*  ****M.H: *M. haemolytica*
Table (3): Potency test of the prepared vaccines using mouse vaccination challenge system and challenged with local isolates of *P. multocida* serotype A:

<table>
<thead>
<tr>
<th>Groups</th>
<th>LD50 7th days after challenge</th>
<th>Log protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>10^{1.96}</td>
<td>5.24</td>
</tr>
<tr>
<td>Group 2</td>
<td>10^{2.58}</td>
<td>4.35</td>
</tr>
<tr>
<td>Group 3</td>
<td>10^{3.2}</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>10^{7.2}</td>
<td>0</td>
</tr>
</tbody>
</table>

1st Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Montanide™ 01 gel
2nd Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Montanide (ISA 206)
3rd Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Mineral oil

Table (4): Viable counts in mice liver challenged with locally isolate of *M. haemolytica* serotype A1:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Counts in liver mice at hours post challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hrs</td>
</tr>
<tr>
<td>Group 1</td>
<td>5.4±0.80</td>
</tr>
<tr>
<td>Group 2</td>
<td>5.7±0.12</td>
</tr>
<tr>
<td>Group 3</td>
<td>5.8±0.14</td>
</tr>
<tr>
<td>Control</td>
<td>5.6±0.11</td>
</tr>
</tbody>
</table>

1st Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Montanide™ 01 gel
2nd Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Montanide (ISA 206)
3rd Group: Vaccinated with inactivated pneumonic pasteurellosis vaccine adjuvanted with Mineral oil
Control: un-vaccinated

This result is supported by the findings of (Youssef et al., 2005) who stated that mice can be used instead of calves for evaluation of *M. haemolytica* vaccine and determination of the relative immunogenicity of prepared vaccines. In addition, the results of mouse protection test were correlated with the protection offered by this vaccine in calves.

These results indicate that it is possible to modulate the vaccine safety and potency through adjuvant selection and to improve traditional vaccine formulation and it could be recommended to use Montanide™ 01 gel and Montanide ISA 206 as adjuvants to pneumonic pasteurellosis vaccine instead of Mineral oil.

CONCLUSION

The purpose of this study is to compare different adjuvants effect on pneumonic pasteurellosis vaccine and the result of this study suggest using Montanide 01 gel and Montanide ISA206 to enhance the vaccine potency aiming to reach the highest possible protective rate.

CONFLICT OF INTEREST

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

ACKNOWLEDGEMENT

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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