American Journal of Animal and Veterinary Sciences

Original Research Paper

Attempts to Stimulate Immunology Responses by Intramuscular and Intraperitoneal Delivery of EPS-Adjuvanted Mannheimiosis Vaccine

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Article history
Received: 22-06-2022
Revised: 15-11-2022
Accepted: 22-11-2022

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Abstract: This study was conducted on 48 male Sprague Dawley rats to determine the immunological responses of experimental adjuvanted vaccines of *Mannheimia haemolytica* A2 (*M. haemolytica* A2) injection and to observe their protection level upon live *M. haemolytica* A2 challenge. Forty-eight clinically healthy Sprague Dawley rats were divided into four groups; Groups 1 and 2 were vaccinated intramuscularly and intraperitoneally, respectively, with an Exo-Polysaccharide (EPS)-adjuvanted vaccine prepared from formalin-killed M. haemolytica serotypes A2, Group 3 with formalin killed *M. haemolytica* seed. At the same time, Group 4 received intraperitoneally Phosphate Buffer Saline (PBS) as a control. After the first vaccination dose, Groups 1,2, and 3 showed a gradual increase in IgM, IgG, and IgA levels significantly ($p<0.05$). However, their level started to decline six weeks post-vaccination, indicating that the booster dose was needed. Upon the delivery of the second booster dose, antibodies titer showed a persistent increase significantly ($p<0.05$), especially the serum IgG level. All groups were challenged with live virulent *Mannheimia haemolytica* A2 after the level of antibodies declined twice after the second booster was delivered. No rat deaths were found in the Combined EPS - *M. haemolytica* adjuvant vaccine after 14 days following the challenge (0%). In unvaccinated rats, higher mortality and morbidity were noted (100%) and less was reported in rats receiving *M. haemolytica* A2 Vaccine seed (8%). Histologically at two weeks, post-challenge revealed negligible lung lesions in groups 1 and 2 and mild lesions in group 3. Lung lesions were recorded in all unvaccinated control rats. Furthermore, *M. haemolytica* A2 re-isolated successfully from lung samples in groups 3 and 4. In conclusion, Rats receiving adjuvant-*M. haemolytica* vaccine confers reasonably high preventive efficacy compared to *M. haemolytica* A2 by itself. Further studies should be conducted on measuring the antibody titer in different vivo, such as goats or sheep.

Keywords: Adjuvant, Antibody, Immunology, Mannheimiosis, Vaccine

Introduction

Pneumonic Mannheimiosis (PM) is one of the most frequently respiratory bacterial diseases among small ruminants throughout many countries, including Malaysia's production animals. PM is responsible for huge economic losses in calving breeding in the USA (Capik *et al*., 2021). The similarity is seen in Europe (Avalos-Gómez *et al*., 2020). In addition, PM was reported in Malaysia in the early 1800's infected cattle and buffaloes (Chandrasekaran *et al*., 1994). PM caused financial losses due to mortality, healthcare costs, and decreased productivity over time (and likely a lower grade of meat in cattle treated for the disease several times).

PM which caused by a gram-negative pathogenic bacterium known as *Mannheimia haemolytica* (*M. haemolytica*). *M. haemolytica* causes bacterial respiratory mortality in pigs, sheep, and goats, as well as mastitis in camels and ewes and, on rare occasions, abortion in cattle. It has also been reported that it was isolated from domesticated birds (Alley, 2002; Camacho *et al*., 2005; Erfan, 2013; Kaneene and Scott Hurd, 1990; Legesse *et al*., 2018). *M.*

haemolytica, in particular, is the causative agent of bovine pneumonic pasteurellosis (older name) or mannheimiosis. *M. haemolytica* is carried by healthy animals as a nasal and nasopharyngeal commensal without causing clinical symptoms. *M. haemolytica*, on the other hand, replicates and is inhaled into the lower respiratory tract of goats (significantly younger animals) when stressed and infected with respiratory viruses (for example, when transported from pastured herds to feedlots).

Mannheimiosis is caused by one of two strains of *M. haemolytica* designed as biotype A serotype 1 in cattle and biotype A serotype 2 in sheep and goats (Kabeta *et al*., 2015). Clinical indicators include fever, cough, nasal discharge, weight loss, and more in both domestic and wild animals (Al-Ghamdi *et al*., 2000; Lawrence *et al*., 2010; Legesse *et al*., 2018). The illness may progress quickly enough to be lethal (Rice *et al*., 2007).

To overcome this problem, vaccination is now considered the most effective and practical therapeutic application for controlling the spread of this infectious disease (Oppermann *et al*., 2017). Vaccination of formalized bacteria has been performed with dubious success for almost 60 years against related respiratory diseases (Rice *et al*., 2007). *M. haemolytica* vaccinations may be improved by recombinant surface augmentation or secreted proteins.

The ability of polysaccharides and other cationic polymers to regulate the release of antibiotics, medicines, or vaccines has lately been exploited in pharmaceutical research and industry (Masotti and Ortaggi, 2009). Additionally, they have received much research as non-viral DNA carriers for gene therapy. Various systems, including poly-lysine and its conjugates, dextran spermine polycations, Polyethyleneimine (PEI), polyamidoamine dendrimers, lipopolyamines, and chitosan, were created in recent years (Masotti and Ortaggi, 2009).

Exopolysaccharides (EPS), produced by algae, has recently attracted a lot of attention due to the possibility that they could be useful compounds and have been used in the field of medicine (Ketabi *et al*., 2008). Exopolysaccharide (EPS) refers to any polysaccharide found outside a microorganism's cell wall. Furthermore, EPS is one of the most important functional components of algae exo-metabolic compounds, which have been found to perform a variety of physiological functions, including immunoregulatory effects. Du *et al.* (2017), antioxidant activities (Lordan *et al*., 2011), anti-hypertensive effects (Borowitzka, 2013), and anticancer activities. Among these, immunoregulatory activity (adjuvant) has recently garnered disproportionate attention.

Many improvements to existing delivery and novel carrier systems are currently being investigated to improve vaccine efficiency. Furthermore, the route of immunization can affect the outcome of the immune response by changing the interaction between the vaccine and distinct APCs at the injection site. As a result, delivery routes influence the therapeutic response by modifying the immunological pathway (Bolhassani *et al*., 2011).

Adjuvant-mediated increase of cellular activation, antigen absorption, and accumulation of antigen cells in the muscle and, later, the lymph node are critical immunological events that prepare for antigen presentation and the formation of antigen-specific immunity (Liang and Loré, 2016).

Vaccine potency and efficiency are usually estimated by antigen-specific antibody titers and T-cell responses generated weeks after vaccination. However, the series of innate immune responses occurring between vaccine delivery and the measurable adaptive immunity induction remain largely unknown (Liang and Loré, 2016).

Materials and Methods

Ethical Approve Statement

All experimental methods and overall protocol were approved by University Malaysia Terengganu (UMT) animal ethics committee with reference number UMT/JKEPHT/2019/1 before the experiment. All attempts have been made to alleviate the suffering of animals.

Study Animals

This study was conducted at the Institute of Marine Biotechnology (IMB), UMT, Malaysia. Forty-eight male Sprague Dawley rats, aged 6-9 months with an average weight of 180-240 g/rat, were purchased from Akrif Bestari Enterprise, Seri Kembangan Selangor, without any vaccination history against Mannheimiosis were selected for this experiment. Rats were individually examined upon reaching the experimental station. Their general health, including body condition and clinical signs such as nasal discharge, coughing, and weakness, was evaluated daily. The study was started once the rat's adaptation period was finished, which was after two weeks.

In four experimental groups, rats were randomly allocated into cages, twelve in four in each group, in identical environmental conditions and housed in ventilated cages at a particular pathogen-free inside the animal house at University Malaysia Terengganu.

Preparing Experimental Adjuvant and Vaccine

The vaccines for this experiment were prepared at IMB Microbiology Laboratory, UMT, Malaysia. Based on their highest isolation rate from various places in Malaysia, *M. haemolytica* A2 was picked and used as a candidate vaccine strain (Effendy *et al*., 1998; Ii, 1987; Jesse *et al*., 2019). This *M. haemolytica* serotype A2 was stored at an IMB germ bank in deep freeze at -80°C. Using Tryptose Soy Broth (TSB), *M. haemolytica* was cultured and grown. The tubes used for culturing go under sterility test by checking the contamination of the tubes incubated for 48 h before using it and the tubes with no growth were used. It was subcultured and incubated at 37°C for 16 h in the broth. The presence of turbidity tested growth. *M.*

haemolytica A2 was harvested from TSB and cultured on Tryptose Soy Agar (TSA) and blood agar. The primary identification procedures were the colony morphology, Purity, hemolysis pattern, and Gram stain results. Vitek 2® System compact (BioMériuex SA, USA) was used to identify the bacteria type. Culture plates were growing at 37°C in a rotatory shaking incubator for 16 h as reported by Brogden *et al*. (1998); Purdy *et al*. (1997). About 30 colonies of the pure bacteria were transferred into 250 mL of Brain Heart Infusion broth (BHI) (Oxoid). The culture was then shaken using a shaker incubator (Labwit Scientific, Australia) and incubated at 37°C for 18 h for replications. Following incubation, serial dilutions and standard plate counting were done to determine the concentration of the culture. *M. haemolytica* A2 cells were then killed by adding buffered formalin 1% (0.25 /25 mL) (Sigma, USA) in Phosphate-Buffered Saline (PBS), overnight and incubated at 4°C. next, the bacterial cells were washed three to five times using PBS (pH 7.4) by centrifugation at 6000 × g for 15 min using refrigerated centrifuge CF16RXII (Hitachi Koki Co. Ltd., Japan) to eliminate the remaining formalin from the cultures, the bacteria were washed again five times with sterile phosphate buffered saline and the concentration in preparation was adjusted to 10^6 CFU/mL using an optical density.

Construction of Adjuvanted Vaccine (Exopolysaccharide - Mannheimia Haemolytica A2 Vaccine)

1 mg of extracted *Tetraselmis Chui* EPS powder was extracted as described by Eppstein *et al*. (1989); Liu *et al*. (2016) and diluted in 100 mL of Phosphate-Buffered Saline (PBS) to prepare a stock solution with a final concentration of 0.01 mg/mL. Then only 50 µL of diluted EPS was taken and mixed with the pellet of 10^6 CFU/mL of formalin-killed *M. haemolytica* A2 (MHA2) vaccine to make exopolysaccharide-*M. haemolytica* A2 vaccine (Kamaradin *et al*., 2021).

Vaccination Experimental Design

The dosage of the vaccine is a constant dose (Nave *et al*., 2019). In the first step of the study, rats from group 1 and group 2 were injected intramuscularly and intraperitoneally, respectively, with 0.5 mL 1×10^6 CFU/mL of formalin-killed *M. haemolytica*-exopolysaccharide adjuvant. Group 3 was injected with a vaccine composed of formalin-killed *M. haemolytica* serotype A2 and Group 4 was injected intraperitoneal with PBS (unvaccinated group), as described in (Table 1).

Challenge Infection

Rats were anesthetized using the mixture of ketamine/xylazine 100 cocktail drugs (mL/kg) via intramuscular injection before the challenge. For vaccinated rats, 16 weeks after vaccination, antibodies start to decline, then rats from groups 2,3, and 4 go under challenge intraperitoneal with 0.5 mL containing 1 × 106 CFU/mL of virulent *M. haemolytica* A2. The same goes; after 16 weeks for group 1, rats were challenged intraperitoneally by 0.5 mL inoculums containing 1 × 106 CFU/mL of live *M. haemolytica* A2. As shown in Table 1. Before the surviving rats were slaughtered on day 14. After the challenge, all rats were observed for any respiratory infection and death symptoms. The entire respiratory tract was examined and the extent of the lung lesions was recorded according to the technique described by Effendy *et al*. (1998); Rice *et al*. (2007).

Isolations of the M. Haemolytica A2

At the time of the euthanizing, organs such as the heart, lungs, liver, and blood were collected. For *M. haemolytica* isolation, samples from the specimens were processed. Samples from which *M. hemolytica* A2 could not be isolated until it was re-cultured up to three times were considered negative and discarded. The suspected Representative colony is recognized as *M. haemolytica* A2 by Vitek 2® System compact (BioMériuex SA, USA). No growth Plates were reincubated for 48 h and discarded if negative.

Statist Determination of Antibody Titers

Blood samples were obtained weekly from each anesthetized Sprague Dawley by tail vein (or trunk blood at postmortem) for immunogenicity testing till the end of the study period, 19 weeks. Blood samples Left to clot at room temperature were centrifuged for 10 min at 8,000 × g. The serum was removed to a new cap tube and processed at -30°C. Enzyme-Linked Immunosorbent Assay (ELISA) was used for Immunogenicity evaluation as described below.

ELISA titer; In rat serum samples, the immunoglobulin (IgM, IgG, IgA) concentration was calculated using an ELISA titer technique similar to the previously mentioned (Effendy *et al*., 1998). Plates were coated with *M. haemolytica* A2 suspension and blocked the remaining protein-binding sites in the coated wells using a blocking buffer. Next, rat serum was added. The detection agent, goat anti-mouse IgM horseradish peroxidase conjugate (Santa Cruz Biotechnology, USA), goat anti-rat IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology, USA), and goat anti-mouse IgA horseradish peroxidase conjugate (Santa Cruz Biotechnology, USA), was added last. At room temperature, each step was incubated for an hour and finished by inverting the plates, shaking the solution in the wells, and removing unbound material by washing the plate. Finally, adding the chromogenic agent and incubating for 30 min at 37°C, read the plate by ELISA microplate reader (Thermo-Scientific Multiskan Ascent TM, USA) at the absorbance of 450 nm.

Table 1: Vaccination experimental design

Group	Rats number	Dosage (mL)	Site and type	Challenge type
1	12	0.5 mL	Adjuvanted (I.M)	Live *M. haemolytica* A2
2	12	0.5 mL	Adjuvanted (I.P)	Live *M. haemolytica* A2
3	12	0.5 mL	formalin-killed *M. haemolytica* A2	Live *M. haemolytica* A2
4	12	0.5 mL	PBS	Live *M. haemolytica* A2

Tissue Processing and Histopathological Assessment

Both unvaccinated and vaccinated rats were sacrificed by general anesthesia 14 days after the challenge was administered. Microscopic investigation of the rats' lungs was carried out by conducting autopsies. Extraction of the entire lung mass was performed without blood transfusion and the lungs were then filled with 10% Neutral Buffered Formalin and inflated (NBF). For paraffin embedding, lungs and other tissues were fixed in 10% NBF for 24 to 72 h and trimmed. Trimmings were made to the right side of the apical lobe of the lungs. A board-certified veterinary pathologist sectioned paraffin-embedded tissues at 5 m thickness and stained them with hematoxylin and eosin for histological investigation.

Statistical Analysis

To evaluate immunoglobulin titers, repetitive measure one-way Analysis of Variance (ANOVA) was used and a Tukey was used to allow pair comparisons between vaccine weeks and groups. Using the Student's t-measure, antibody titers against *M. haemolytica* A2 were evaluated. The findings were evaluated using GraphPad Prism, version 8.0, and a P-value <0.05 was assumed to be significant.

Results

Clinical Observation

Before vaccination, all rats were examined for any pneumonic symptoms. All rats were found negative. All rats were monitored daily after vaccination and after the challenge and clinical signs were recorded. Death and Tachypnoea of the unvaccinated rats after 48 h post-challenge were the notable clinical symptoms in very sick rats. Moderately infected vaccinated rats demonstrated Anorexia, cough, mucoid nasal discharge, and dullness. In this experiment, mortality was observed within 72 h after the challenge (100%) in the unvaccinated control group, while deaths from vaccinated rats (Group 3) were identified within 7 days after the challenge (8%). No rat deaths were found in group 1 and group 2 after 14 days following the challenge (0%). In unvaccinated rats, higher mortality and morbidity were noted and less was reported in rats receiving *the M. haemolytica* A2 vaccine, without death in Combined EPS - *M. haemolytica* A2 adjuvant vaccine. 17 (35%) of the overall 48 rats challenged by live *M. haemolytica* A2 recorded the presence of lesions in the lung, as seen in Table 2.

Microbiological Isolations

M. haemolytica A2 was successfully re-isolated from all pneumonic lungs and lungs without pneumonic lesions did not develop *M. haemolytica* A2. also, re-isolated from all pneumonic lung lesions in both groups 3 and 4, respectively. *M. haemolytica* A2 was isolated with a mean titer range of 10^2-10^6 CFU/g from the lung lesions.

Vaccinated Rats Possess High Serum Titers Against M. Haemolytica A2

Indirect ELISA revealed that rats in the vaccinated group developed substantially higher antibody titers than the unvaccinated group after vaccination. (P<0.0001). Pre-vaccinated, an indirect ELISA revealed that all rats had low serum antibody titers against *M. haemolytica* A2. In contrast with the control group, serum antibody titers against *M. haemolytica* A2 increased dramatically in all vaccinated rat groups following vaccination. (P<0.0001) (Fig. 1). Between the first vaccination and the following second vaccination(booster), a large rise in antibodies was also recorded (P<0.05). The antibody titers against *M. haemolytica* A2 were initially poor for vaccinated groups but began to rise in groups 1, 2, and 3 as early as four weeks after the first vaccination and peaked in group 1 after seven weeks and in group 2 at week six. The peak level of group 1 and 2 antibodies was significantly (P<005) higher than that of group 4 unvaccinated controls. For group 4, the antibody titers remained low and the variations between weeks were not important at most intervals.

Histology of the Bronchus-Associated Lymphoid Tissue (BALT)

This was done as described by Zamri-Saad and Effendy (1999). The exposure of the antigenic substances via intraperitoneal and intramuscularly administration routes that were applied did not only give results on the humoral responses by the development of antibody production in the systemic compartment but also provided us with valuable insight into the cellular responses of morphological changes in the respiratory tracts in terms of the histological lesion of the lung tissue, the size area (μm^2) of Bronchial-Associated Lymphoid Tissue (BALT) and the number of lymphocytes infiltrated within the bronchial area (Emikpe and Ajisegiri, 2011).

In this study, the lung of the right apical lobe, which is the first route of the lung to be exposed to pathogens, has

been chosen and processed for histology from all groups following vaccination of the EPS-MHA2 vaccine and MHA2 vaccine. Two weeks after the challenge, the entire lung of the right apical lobe from all groups had been sectioned at 5 µm. Paraffin sections were stained with Haematoxylin and Eosin (H&E). The 5-site formation of the development of BALT around the bronchus and bronchi was selected and examined under the 5x and 20x objective lenses of the light microscope. The development of BALT in lung tissues was notified of the dark purplish-blue color and the BALT structure observed around the wall of the bronchus and bronchioles. Representative lung sections from the PBS group (Fig. 2), as well as sections from the lungs of rats vaccinated with the MHA2 vaccine (Fig. 3b) and EPS- MHA2 vaccine (Fig. 2), is reported. Histopathological analysis of sections revealed the presence of the BALT. group showed a severe lesion of Pneumonic mannheimiosis with thickened interalveolar space. The other two groups showed a less histological lesion of Pneumonic mannheimiosis; meanwhile, the EPS-MHA2 vaccine group showed more formation of BALT than others. Type of formation aggregates. The area of bronchus or bronchioles rounded with aggregations of lymphocytes without suppression, the mucosal epithelium is categorized as an aggregates type of BALT and always appears in association with the bifurcation of the major bronchus (Effendy *et al*., 1998).

Table 2: Clinical observation of sprague dawley rats

Group	Vaccine	Bacterial Challenge	Challenged rats	Sick rats	Rats with lung lesions	Dead rats	Isolation of *M. haemolytica* A2
1	Adjuvanted (I.M)	Live *M. haemolytica* A2	12	1 (8%)	1(8%)	0 (0%)	Nil
2	Adjuvanted (I.P)	Live *M. haemolytica* A2	12	2 (16%)	1 (8%)	0 (0%)	Nil
3	formalin-killed *M. haemolytica*	Live *M. haemolytica* A2	12	5 (42%)	3 (25%)	1 (8%)	Lung only
4	PBS	Live *M. haemolytica* A2	12	12 (100%)	12 (100%)	12 (100%)	Lung only
	Total	---	48	20 (42%)	17 (35%)	14 (29%)	---

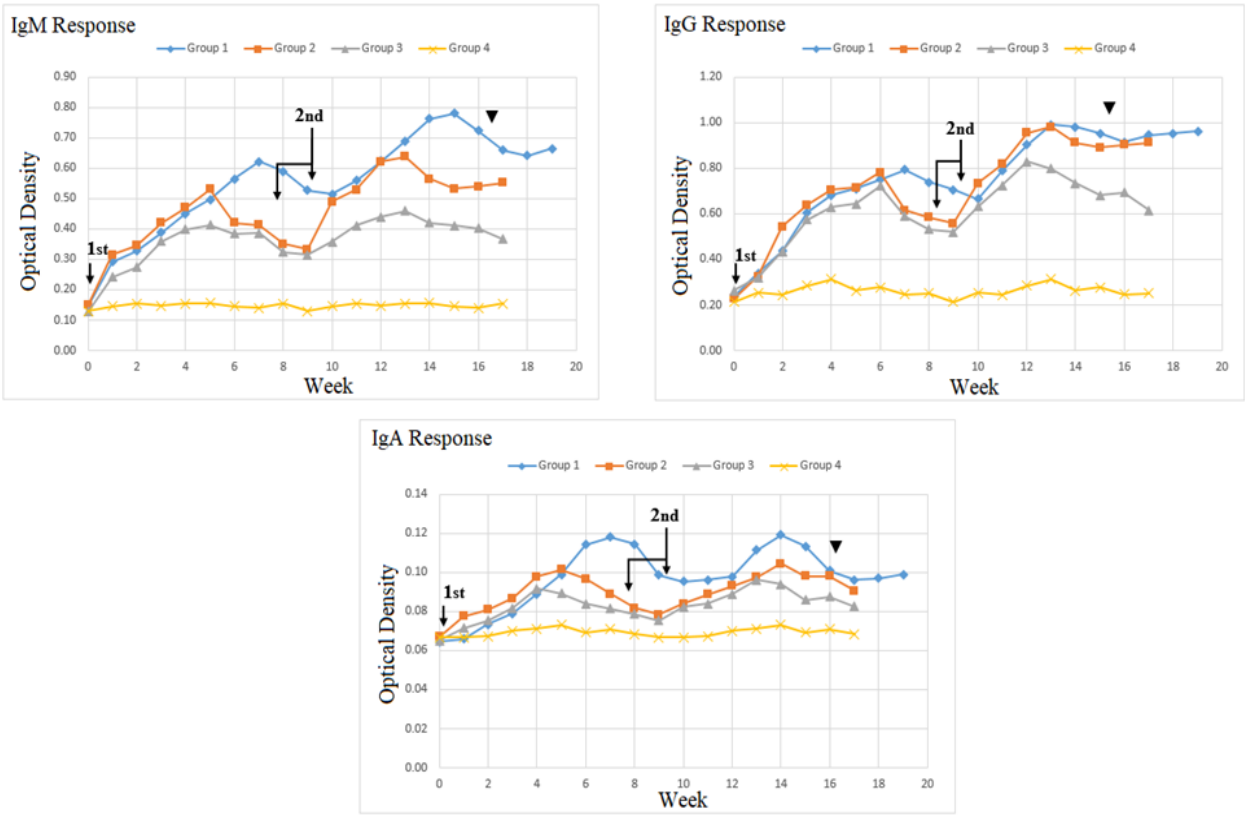

Fig. 1: Serum IgM, IgG, IgA Antibody levels in rats. Group 1; intramuscular vaccination. Group 2; intraperitoneal vaccination. Group 3; *is M. hemaolytica A2* vaccination and Group 4 is; the unvaccinated control group. Arrows represent vaccine injection and the arrowhead poses a challenge with live *M. haemolytica* A2

The Size of Bronchus-Associated Lymphoid Tissue (BALT) and Lymphocyte Number Within the BALT

Other than reviewing the histological lesion in the lung tissues, the differences in BALT responses should be highlighted by calculating the average size area (μm^2) of BALT formation and the number of lymphocytes formed within BALT using the software LAS 4.0 system. Based on Fig. 3 (a), the average size of BALT showed an increasing size of BALT in the EPS-MHA2 vaccine group (361689 μm^2), MHA2 vaccine group (155672 μm^2), and PBS group (80246 μm^2). However, when focusing on comparing the average size of BALT formed between groups, the group that was vaccinated with the MHA2 vaccine showed a significant ($p<0.05$) high in the average size of BALT when compared with the PBS group; meanwhile, the EPS- MHA2 vaccine group was showing a significant ($p<0.05$) high when compared with those from MHA2 vaccine group and PBS group. Even so, the EPS-MHA2 vaccine group is still regarded as the highest in BALT formation compared to the other two groups.

Focusing on Fig. 3, the development pattern seems to differ in the average number of lymphocytes. Only the EPS-MHA2 vaccine group (4500) showed a significantly ($p<0.05$) highest average number of lymphocytes when compared to the MHA2 vaccine group (1109) and PBS group (500).

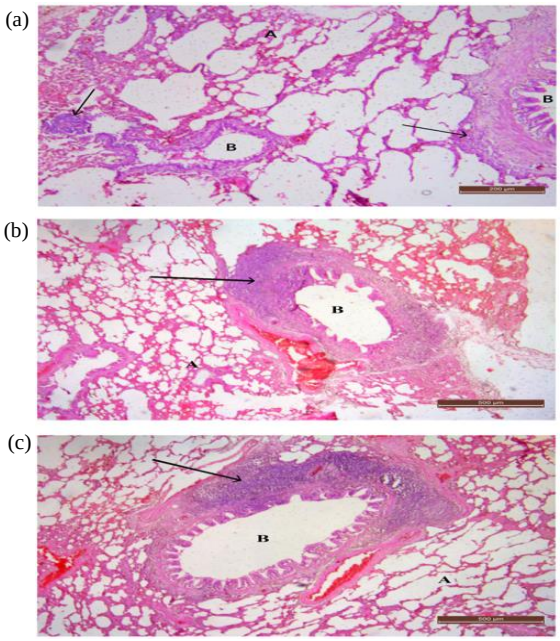

Fig 2: Photomicrograph of lymphoid aggregates (arrow) that were stained with Haematoxylin and Eosin (H&E) and formed in the lung of rats treated with (a) PBS group (b) *M. haemolytica* A2 vaccine group (c) Exopolysaccharide-*M. haemolytica* A2 vaccine group. A: Alveolar space; B: Bronchiole. Total magnification 4x and 50x (Scale bar = 500 and 200 μm)

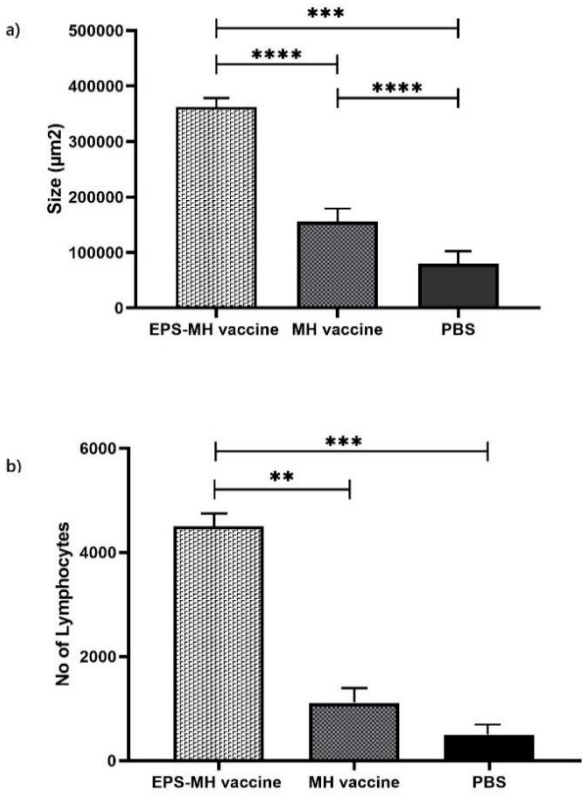

Fig 3: (a) Comparison of the size of BALT area between PBS: Phosphate Buffered Saline group, MHA2: *M. haemolytica* A2 vaccine group, and EPS-MHA2: EPS-*M. haemolytica* A2 vaccine group. (b) Comparison of the number of lymphocytes between PBS: Phosphate buffered saline group, MHA2: *M. haemolytica* A2 vaccine group, and EPS-MHA2: EPS- *M. haemolytica* A2 vaccine group occurred in the BALT area. Values are means ± SEM, (n = 3) goats per group; *p<0.05, ***p<0.001, ****p<0.0001; One-way ANOVA was performed, followed by Tukey's multiple tests for analysis

Discussion

Vaccination is the widely accepted method of controlling PM. Vaccines of various types are used. Simple bacterins only provide immunity for a few weeks. Many countries use the Oil Adjuvant Vaccine (OAV), which provides both a higher level and a longer duration of immunity (l year) and is thus the most well-established. Since described this vaccine, research on various vaccines has been conducted and the search for a better vaccine continues.

Edible, adjuvant-based vaccines have been studied since the early 1990s. Many characteristics make adjuvants an appealing medium for disseminating vaccine antigens (Di Pasquale *et al*., 2015). When an antigen is paired with an adjuvant, immune response production may be substantially stimulated, thus improving its availability and usefulness for humans and animals.

Chang *et al*. (1998) exopolysaccharides act like antigen-keeper bioreactors and have a typical built-in antigen encapsulation system (Liu *et al*., 2016). Literature reviews show that antigen immunogenicity and biological processes are at the highest degree when delivered with an adjuvant due to their normal antigen encapsulation. Wang and Singh (2011), bioencapsulation has been shown to impair the extended release and appearance of the antigen for immune receptive sites (Riccio and Lauritano, 2020; Xiu *et al*., 2018).

Therefore, this experiment was planned to test the hypothesis that vaccination using an adjuvanted vaccine will generate high antibodies against *M. haemolytica* A2 and protect vaccinated rats upon challenge with live *M. haemolytica* A2. The observation and study of EPS, which acquires immunostimulatory activity and increases the immune response (Wu *et al*., 2010; Xiu *et al*., 2018), created our hypothesis.

This adjuvanted vaccine forms homogeneous structures, the composition of exopolysaccharide and formalin-killed *M. haemolytica* A2 able to proliferate and activate macrophage cells (unpublished data). Therefore, to potentiate cellular immune and humoral antibody responses against *M. haemolytica* A2, EPS has acquired interest as a carrier victor.

The results collected in this study clearly show that when Exopolysaccharide (EPS) is combined with the antigen in the vaccine, significant protection can be obtained. The administration of EPS allowed the content of *M. haemolytica* A2 antigen to be condensed and standardized in vaccine doses.

In this experiment, the authors observed intraperitoneal vaccination of Sprague-Dawley rats by adjuvanted vaccine expressing less antibody response against the live *M. haemolytica* A2 challenge. On the other hand, intramuscular vaccination showed a much higher response upon challenge with live *M. haemolytica* A2. These enterally vaccinated rats showed strong IgG, IgM, and IgA responses for more than six sequential weeks after the first vaccination, with a gradually elevated level of IgM, IgG, and IgA antibody production against *M. haemolytica* A2 seen in all the weeks, especially after the second vaccination (booster) injection in each vaccinated rat. Furthermore, total postmortem lung lesions and bacteriology differed among vaccinated and control rats. The authors suggest that rats with lung lesions of more than 25% are likely to succumb to the disease, whereas rats with less than 25% are likely to rebound.

Based on the significant values of the average size of BALT and the number of lymphocytes, the group that was vaccinated with the EPS- MHA2 vaccine had induced a significant ($p<0.05$) increase in the size of BALT area as well as in the number of lymphocytes within BALT when compared with MHA2 group and PBS group. This observation proved that the local immune responses were successfully stimulated following the vaccination of the EPS-MHA2 vaccine, which was regarded as antigenic stimulation for the formation of the lymphoid aggregates at the bronchus, which is later capable of producing the mucosal secretory antibody in protecting the mucosal membrane of the lungs from further colonization of *M. haemolytica* A2 (Effendy *et al*., 1998; Emikpe and Ajisegiri, 2011; Ezeasor *et al*., 2013; Zamri-Saad *et al*., 1999). This result follows most researchers' findings who reported that the morphologic and hyperplastic changes in the BALT resulted from antigenic substances stimulation or lung infection (Effendy *et al*., 1998; Zamri-Saad *et al*., 1999; Emikpe and Ajisegiri. 2011; Ezeasor *et al*., 2013).

Therefore, the findings recorded in this study on exopolysaccharide vaccination as an adjuvant in rats consider the basis for developing similar *M. haemolytica* A2 vaccines in larger animals. At the same time, various reports document the immunogenicity of nasal adjuvanted vaccines given in goat animals (Effendy *et al*., 1998). The concept of adjuvanted vaccines against mannhmosis has been successfully generated by Effendy *et al*. (1998) in their study on EPS- *M. haemolytica* vaccine antibody responses against *M. haemolytica* A2 challenge in goats following nasal spray immunization with EPS-adjuvanted vaccines, adjuvanted spray vaccine yield antigen-specific immune responses in the mucosal system of the goats resulted from the delivery (unpublished work). These results suggest that the nasal-administered adjuvanted vaccine facilitates the idea and builds the next step of the vaccination process to include innate and systemic immunity with a different site of administration.

Conclusion and Future Work

Improved adjuvant selection is one of the recent developments aimed at enhancing vaccine potency. As a result, better vaccines for prophylaxis and treatment can be advanced by creating more effective and safer adjuvants.

The role of exopolysaccharide as an adjuvant vaccine candidate must be studied in developing a new generation vaccine for controlling PM in goats. Moreover, with the advance in biotechnology and understanding of EPS agents combined with the knowledge of the host immune response, the production of antibodies against *Mannheimia haemolytica* A2 through an adjuvanted vaccine would be advantageous under a variety of circumstances.

Future work is required to bring the final result of exopolysaccharides to help reduce Mannheimiosis in goats. An experiment of immunoglobulin levels by time upon adjuvanted vaccination is currently in process in our lab; Data over 13 weeks show that the response has improved and could last in goats for several months. It is also challenging to determine how well a vaccine will perform from non-finished results. Further feasibility evidence may be provided by experiments using models such as rats to endorse initial finding trials. Altogether,

these findings provide the rationale for pursuing the continued invention of vaccines to treat and prevent Mannheimiosis disease.

In conclusion, our preliminary findings have shown that EPS is a safe and efficient novel adjuvant injected intramuscularly and can potentially be used for sub-unit vaccine production. The process of EPS should be further studied in larger experimental groups to consider the potentiality of EPS as a novel adjuvant.

Acknowledgment

The authors would like to thank to institute of marine biotechnology, university Malaysia Terengganu, and all the research assistants for all their technical assistance.

Funding Information

This study was funded by the Science and Technology Research Partnership for Sustainable Development (SATREPS), Japan International Cooperation Agency (JICA), Japan Science and Technology Agency (JST), and the Ministry of Higher Education Malaysia (MOHE) under Vot No. 53220.

Author's Contributions

Ghaith Hussein Mansour: Performed experiments, collect date and perform statistical analysis.

Laith Abdul Razzak: Analyzed each step of the experiment and interpreted date.

Hassan Ibrahim Sheikh: Wrote the paper and perform statistical analysis.

Mohd Effendy Abd Wahid: Conceived and designed the experiments, contributed reagents, materials, analysis tools.

Ethics

All experimental methods and overall protocol were approved by the UMT animal ethics committee with reference number UMT/JKEPHT/2019/1 before the experiment. All attempts have been made to alleviate the suffering of animals.

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