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Review Article

Potential of *Chlorella* sp. exopolysaccharide as adjuvant for *Mannheimia haemolytica* A2 vaccine in rat model

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Abstract

The potential of exopolysaccharides (EPS) extracted from *Chlorella* sp. as adjuvant for formalin-killed whole organism of *Mannheimia haemolytica* A2 (MhA2) vaccine was investigated. *Chlorella* sp. was cultured in F/2 media and EPS was extracted and characterized using GPC, FTIR and SEM, while its cytotoxicity was tested on RAW 264.7. Sprague Dawley rats were inoculated intranasally with either phosphate-buffered saline (PBS) (Group A), EPS only (Group B), formalin killed whole organism of MhA2 vaccine seed only (Group C) or EPS-MhA2 adjuvanted vaccine (Group D). Serum levels of IgM, IgG, and IgA titers were collected for nine weeks. About 4.8 mg of EPS was obtained in the form of white powder with molecular weight of 2398 Da. The microstructure of EPS was compact and exhibited flakes like structural units and was not toxic to RAW 264.7. EPS-MhA2 group produced significantly higher ($p<0.05$) IgM, IgG and IgA responses compared to other groups.

Keywords: exopolysaccharide, *Mannheimia haemolytica* A2, adjuvant, immunoglobulins, intranasal

1. Introduction

Vaccinations are the most effective strategy in the prevention of infectious diseases by generating immune responses and maintain memory cells that are able to induce rapid recall responses (Bartlett & Tyring, 2009; Sarkander, Hojyo, & Tokoyoda, 2016). The entry point of more than 90% of pathogens is via mucosal membrane and mucosal vaccination is an effective approach (Miquel-Clopés, Bentley, Stewart, & Carding, 2019). The vascularized mucosal surface area is 150 cm^3 at the nasopharyngeal compartment and can be targeted for vaccine uptake (Holmgren & Czerkinsky, 2005; Olszewska & Steward, 2001; Wang, Liu, Zhang, & Qian, 2015).

Pneumonic mannheimiosis caused by *Mannheimia haemolytica* serotype A2, a gram-negative commensal bacterium that can be found at the upper respiratory tract of domestic and wild animals including healthy ruminants (Roier *et al.*, 2013). The bacterium can turn opportunistic when the animals are exposed to predisposing factors such as

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pregnancy, transportation or introduction of new animals into an existing herd. Pneumonic mannheimiosis can lead to high mortality and economic losses to the ruminant industry (Jesse *et al.*, 2014).

M. haemolytica initiates their infection at the lining mucosal surfaces of the lower respiratory system (Wang *et al.*, 2015). The efficacy of mucosal vaccination through an intranasal route of delivery had been successful in small ruminants (Effendy, Zamri-Saad, Mohamad, & Omar, 1998a; Effendy, Zamri-Saad, Puspa, & Rosiah, 1998b; Zamri-Saad, Effendy, Israf, & Azmi, 1999a; Zamri-Saad, Maswati, Effendy, & Jasni, 1999b). However, the uptake of vaccines from the nasal cavity is poor due to rapid clearance and poor transportation across the nasal mucosal membrane (Svindland *et al.*, 2012). Therefore, the intranasal administration of formalin-killed whole organism of *M. haemolytica* A2 (MhA2) vaccine would need an extra component, such as an adjuvant that is capable of sustaining their presence and ameliorating the immune responses.

Exopolysaccharide (EPS) is an organic macromolecule that consists of various types of sugars and is produced by many microorganisms. The EPS can be found either as capsular matrix that bound covalently to the cell surface or as slime materials that bound loosely (Ahmed, Wang, Anjum, Ahmad, & Khan, 2013; Badel, Bernardi, & Michaud, 2011). In marine environment, EPS form biofilm, which allows the cells to adhere to other bacteria, animal, plant tissues or inert surfaces (Sutherland, 2001). Due to these properties, EPS could have muco-adhesives character that might be suitable as mucosal adjuvant that could ensure long retention and high absorption rates in the nasal cavity (Svindland *et al.*, 2012; Zaman, Chandrudu, & Toth, 2013).

In recent studies, EPS from marine origin such as microalgae has been used in pharmaceuticals industry due to its immune-modulatory, apoptotic and antiviral activity (Xiao & Zheng, 2016; Ishiguro *et al.*, 2017). Hence, EPS from *Chlorella* sp. was chosen to elucidate its potential as a mucosal adjuvant vaccine for formalin-killed whole organism of *Mannheimia haemolytica* A2 vaccine. The study aims at evaluating the *in vivo* adjuvant activity of EPS using Sprague Dawley rat model to determine whether EPS can enhance the humoral immunity in systemic compartments by examining the development of antibodies (IgM, IgG and IgA) responses.

2. Materials and Method

2.1 Extraction of EPS from *Chlorella* sp.

A pure culture of *Chlorella* sp. ($1x10^6$ CFU/mL) that was cultured into F/2 media at ratio of 1:1, was centrifuged at 15,000 rpm for 20 minutes at 4 ^oC using refrigerated centrifuge in order to extract EPS following previously established method with slight modifications (Bajpai, Majumder, Rather, & Kim, 2016). Then, supernatant produced were mixed with 100% ice-cold ethanol at ratio 1:1 (25 mL/ 25 mL) and incubated at 4 ^oC overnight. After 24 hrs of incubation, the solution was centrifuged again at 15,000 rpm for 20 minutes at 4 ^oC. The pellet was collected and washed twice with distilled water then dissolved in distilled water. The pellet was then dialyzed using 16.5 x 26 mm visking tubing membrane for 24 hrs at 4 ^oC to remove protein residues. Next, the solution in visking tube was transferred into the falcon tube and freeze-dried using vacuum freeze dryer. The dried pellet obtained was weighted and stored refrigerated at -20^oC.

2.2 Identification of EPS from *Chlorella* sp.

2.2.1 Gel Permeation Chromatography (GPC)

The molecular weight of EPS pellet was analyzed using Gel Permeation Chromatography (GPC) method using a Waters 1515 HPLC system equipped with Waters 2414 refractive index detector (RID). Three types of columns, namely 7.8 x 300 mm Ultrahydrogel^{TM} 500 columns, 7.8 x 300 mm Ultrahydrogel^{TM} 120 columns and 7.8 x 300 mm Ultrahydrogel^{TM} Linear columns were used. Then, 1 mg of EPS was dissolved in 1mL of 0.1 M $NaNO_3$ and injected. The eluent was 0.1 M $NaNO_3$ in water. The columns and detector were maintained at 35 ^oC. The sample was analyzed within 40 minutes at a flow rate of 1 mL/min and the injection volume was set at 25 µL. Dextran molecular weight standard kit (Polymers Standards Service-USA, Inc. Germany) was used in the GPC as a standard. The data collection and analysis were carried out using Breeze 2 software (Xing *et al.*, 2017).

2.2.2 Fourier Transform Infrared spectroscopy (FTIR)

The spectra of EPS were recorded using spectrophotometer (Perkin Elmer, USA) using Potassium Bromide (KBr) disc. FTIR absorption band were read at wavelengths (λ) between 2.5 µm to 25 µm and measured in cm^{-1} unit (Siti-Aisha, 2018).

2.2.3 Scanning Electron Microscope (SEM)

The diluted EPS sample (10^6 CFU/mL) was centrifuged at 4,000 rpm for 15 minutes. The pellet obtained was placed on a small circle glass slide and dried in room temperature for 15 minutes. Next, the slide with dried pellet was placed on stubs, then coated with the gold 20-40 µm thicknesses using auto fine coated JFC 1600 and viewed under SEM (Akin & Amos, 1975).

2.3 Cytotoxicity test of EPS

2.3.1 Measurement of cell viability assay by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-Diphenyl-Tetrazolium Bromide (MTT)

Viability of the cell against EPS was determined via 3-(4,5-dimethylthiazol-2-yl)-2, 5-Diphenyl-Tetrazolium Bromide (MTT) assay (Razali *et al.*, 2014). Briefly, RAW 264.7 cells that obtained from the American Type Culture Collection (ATCC) was cultured according to the method described by Kim *et al.* (2015). After the cells culture reached 80% confluence in the culture flask, cells were seeded in 96 wells plate at the density of $5x10^4$ cells/well. After 24 hours of incubation, the adhered cells were treated with various concentrations of the EPS that was diluted in DMEM (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.12 µg/mL, and 1.56 µg/mL, Control positive (DMEM only). Twenty-four hours later, MTT was added and the cells were

incubated for 4 hrs at 37 °C and 5% CO_2. The medium was then removed, and the formazan precipitate was solubilized in DMSO. The absorbance was measured at 550 nm by using microplate reader (Thermo-Scientific Multiskan Ascent^{TM}, USA) for cell viability calculation. The cytotoxicity of EPS was expressed as the IC_{50}, which is the concentration of each EPS that reduces the 50% of cell survival when compared with the control.

Cell viability (%) = (OD_{550} of sample) (OD_{550} of control)$^{-1}$ x 100

2.3.2 Preparation of formalin-killed cells (FKCs) of *Mannheimia haemolytica* A2 (MhA2) vaccine

Mannheimia haemolytica A2 were cultured on Thermo Scientific Columbia Sheep Blood Agar (BA) plate. About 30 colonies of the pure bacteria were transferred into 200 mL of Brain Heart Infusion broth. The culture was then shaken by using a shaker incubator at 37 °C for 18 hrs for replications. Following incubation, serial dilutions and standard plate counting were done to determine the concentration of the culture. The bacteria were then killed by introducing neutral-buffered formalin at the concentration of 1% (0.25 mL/ 25 mL) formalin in phosphate buffered saline and kept overnight at 4 °C. Then the bacteria were washed five times with PBS by centrifugation at 6000 xg for 15 minutes using refrigerated centrifuge to eliminate the remaining formalin from the cultures. The bacteria were washed again five times with sterile PBS, and the concentration in preparation was adjusted to 10^6 CFU/mL using optical density (Noraini, Sabri, & Siti-Zahrah., 2013).

2.3.3 Preparation of exopolysaccharide-*Mannheimia haemolytica* A2 (EPS-MhA2) vaccine

The 4.8 mg of EPS powder were diluted in 400 mL of phosphate buffered saline to prepare as a stock solution with a concentration 0.012 mg/mL. Then only 30 µL of diluted EPS was taken and mixed together with the pellet of 10^6 CFU/mL of formalin-killed *M. haemolytica* A2 (MhA2) vaccine to make exopolysaccharide-*M. haemolytica* A2 (EPS-MhA2) vaccine (Noor-Hidayah, 2014).

2.4. Confirmation of exopolysaccharide-*Mannheimia haemolytica* A2 (EPS-MhA2) vaccine

2.4.1 Scanning Electron Microscope

The mixture of diluted EPS and 10^6 CFU/mL of MhA2 vaccines were centrifuged at 4,000 rpm for 15 minutes using centrifuge. Then, the pellet was collected and placed on small round glass slide and dried in room temperature for 15 minutes. The slide with dried pellet was coated with the gold 20-40 µm thicknesses using auto fine coated JFC 1600 and ready to view in low vacuum condition under SEM (Akin and Amos, 1975).

2.4.2 Experimental design of white rats (Sprague Dawley)

Twelve female healthy Sprague Dawley of 6-8 weeks age were selected and allocated randomly into 4 groups with 3 rats each group assigned as group A, group B, group C and group D. Group A and B were negative control treated with phosphate buffered saline and EPS as adjuvant respectively. Group C was positive control treated with MhA2 vaccine only and Group D was treated with EPS-MhA2 vaccine, which served as an adjuvant vaccine. All Sprague Dawley were inoculated by dripping 30 µL of samples via the intranasal route. Blood samples were taken every week from each of the Sprague Dawley's tail vein for determining the antibody production of the rats. The first treatments were administered into the Sprague Dawley's nostril and labeled as Week 0. Then on Week 2, second booster dose was given. Six weeks after 2nd vaccination (Week 8), all rats were euthanized according to the animal ethics code UMT/JKEPHT/2017/2.

3. Results

3.1 Physical and chemical characterization of exopolysaccharide (EPS) from *Chlorella* sp.

The 400 mL of *Chlorella* sp. culture containing 10^6 CFU/mL had yielded 4.8 mg in the form of white powder that and was readily soluble in water (Figure 1). Gel Permeation Chromatography (GPC) revealed that the average molecular weight (Mw) of the EPS was 2,398 Da as shown in (Figure 2). FTIR spectrum indicates the major functional groups and chemical bonds present in EPS. The peak at 3,350.35cm^{-1} indicated O-H stretch. A weak absorption band at the wave-number region <3,000 cm-^{1} was assigned to the symmetric stretching vibration of CH group. A sharp peak found at 1,683.86 cm-^{1} and 1,402.25 cm-^{1} indicated stretching vibrations of C=O and C-O respectively which are linked to COOH groups. While the absorption bands in the region 1,116.78 cm-^{1} and 975.98cm-^{1} suggested a typical signature of carbohydrates molecules (Figure 3). Morphology of freeze-dried EPS was visualized by Scanning Electron microscope (SEM) analysis at 2,500x magnification. The microstructure of freeze-dried EPS was found to have compact in structure and flakes like structural unit structure with sharp edges (Figure 4).

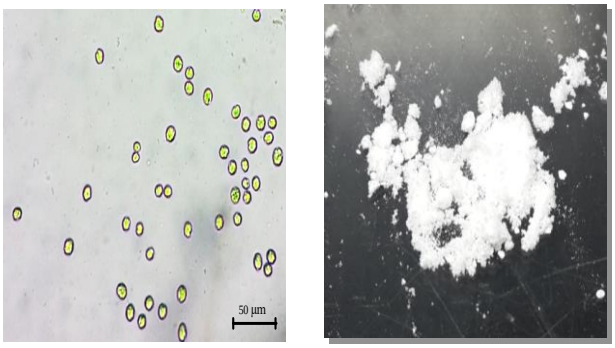
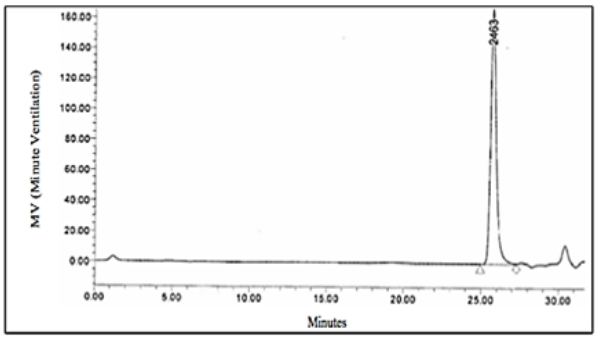


Figure 1. Photomicrograph of a) cultured *Chlorella* sp. used in this study and b) the physical appearance of EPS extracted from cultured *Chlorella* sp. as freeze-dried powder. Total magnification = 400x (Scale bar = 50 µm)



	Elution Volume (mL)	Retention Time (min)	Adjusted RT (min)	Mn	Mw	MP	Mz	Mz+1	Mz/Mw
1	25.744	25.744	25.744	2358	2398	2463	2432	2462	1.014

Figure 2. Chromatogram molecular weight of EPS extracted from *Chlorella* sp. using Gel Permeation Chromatography (GPC)

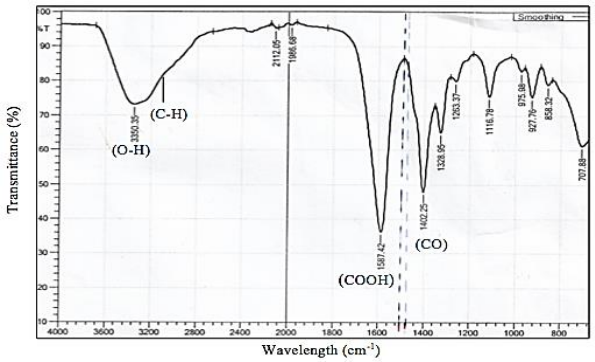


Figure 3. FTIR spectrum of crude EPS extracted from *Chlorella* sp. showing the typical structure and functional groups

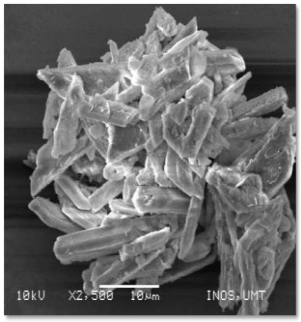


Figure 4. SEM image of freeze-dried powder of EPS extracted from *Chlorella vulgaris*. Total magnification 2,500x (Scale bar = 10 µm)

3.2 Cytotoxicity of exopolysaccharide (EPS) from *Chlorella* sp.

The in vitro cytotoxicity test of EPS was evaluated using MTT assay on Mouse Leukaemic monocyte-macrophages cell line (RAW 264.7). The MTT assay was performed as percentage relative viability of cells (%) versus concentrations of EPS extracts (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.12 µg/mL, and 1.56 µg/mL) and the percentage viability of RAW 264.7 cell was calculated after 24 hours exposure in concentration-dependent manner. The results as shown in Figure 5 indicated that EPS promoted the growth of RAW 264.7 cell at all concentrations (3.125 µg/mL (112.21%), 6.25 µg/mL (125.07%), 12.5 µg/mL (140.34%), 25 µg/mL (143.97%), 50 µg/mL (152.01%), 100 µg/mL (140.84%)) except at this concentrations 1.56 µg/mL (67.24%) of EPS where the RAW 264.7 growth was lower than control (100%). There was no significant differences ($p>0.05$) in percentage relative viability of cell between tested concentrations and the control. No IC_{50} value was obtained; hence, EPS is safe at all tested concentrations.

3.3 Morphology of exopolysaccharide-*Mannheimia haemolytica* A2 (EPS-MhA2) vaccine

In order to confirm whether the EPS powder able to encapsulate whole-cell formalin killed *M. haemolytica* A2, the morphological structure of the EPS-MhA2 was observed under SEM. Based on Figure 6, the clumping structure showed the EPS had successfully encapsulated the whole-cell formalin-killed *M. haemolytica* A2 under 6,1500x magnification.

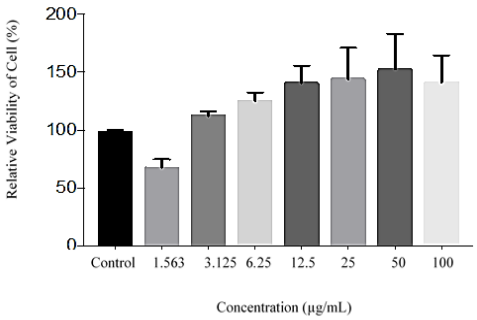


Figure 5. Graphs showed percentage relative viability of RAW 264.7 macrophage cell that was treated with various concentrations of EPS diluted in DMEM (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.12 µg/mL and 1.56 µg/mL). The control group was treated with complete DMEM only. Values are mean ± SEM, (n=3); One-way ANOVA was performed followed by Tukey's multiple comparisons test for analysis.

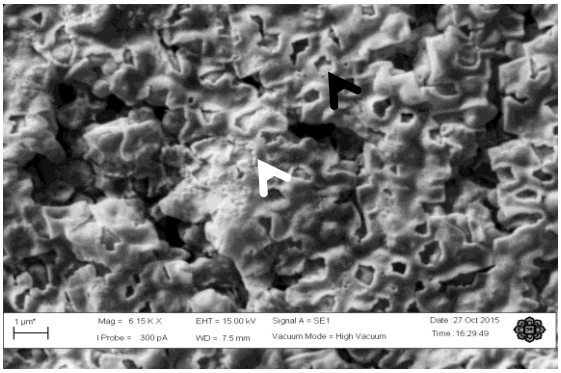


Figure 6. SEM images of exopolysaccharide (EPS) mixed with sterile PBS as excipient able to capsulate *M. haemolytica* A2 vaccine seed. Total magnification 6,1500x (Scale bar = 1 µm). White arrow: Fragment of Exopolysaccharide, Black arrow: *Mannheimia haemolytica* A2 (MhA2) vaccine seed

3.4 Development pattern of IgM, IgA and IgG of white rats (Sprague Dawley)

Enzyme-linked Immunosorbent Assay (ELISA) results revealed that there were three types of response patterns showed by IgM, IgG and IgA antibodies in the blood serum of experimental Sprague Dawley rats before and after vaccination (Figure 7). At the starting week (Week 0) before vaccination program, the levels of IgM, IgG and IgA in all Sprague Dawley were at low with no significant ($p>0.05$) differences between groups. However, following the 1st intranasal vaccination on Week 1, the development of IgM, IgG and IgA responses in all groups of Sprague Dawley showed a different pattern of responses.

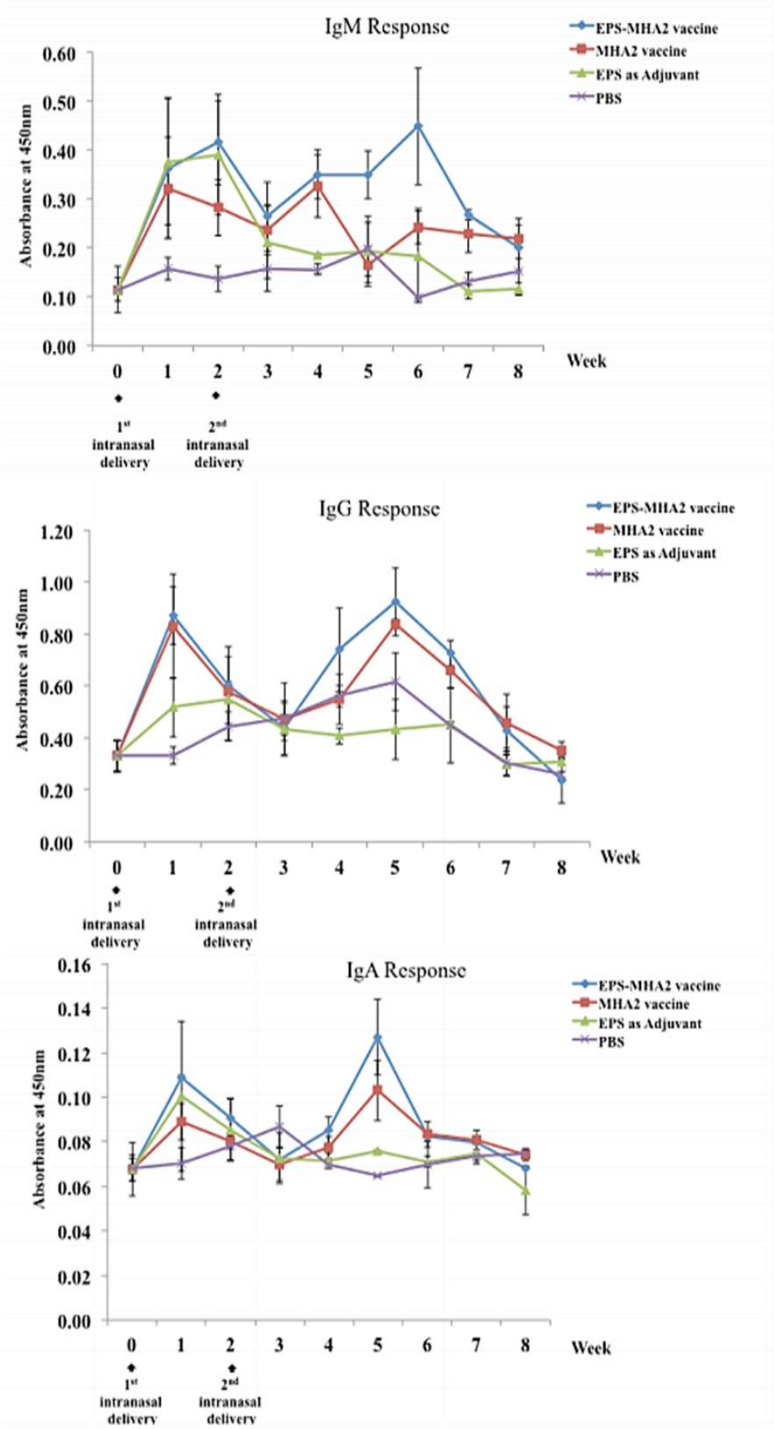


Figure 7. Pattern of responses of IgM, IgG and IgA in Sprague Dawley for each week of experiment. Values are mean ± SEM, (n=3) rats per group; One-way ANOVA was performed using the Tukey's multiple comparisons test for analysis.

For the IgM, after the 1st intranasal vaccination on Week 1, the level of IgM in all groups showed a gradual increase but the increment was not significant ($p>0.05$) until Week 3. However, following a booster dose on Week 2, despite slight decrease at Week 3, both EPS-MhA2 and *M.* MhA2 vaccinated groups showed significant ($p<0.05$) increase at Week 4 when compared with both unvaccinated groups. At Week 5 and Week 6, only EPS-MhA2 group sustained a significant increase ($p<0.05$) when compared to other groups. For IgG, at Week-1 post intranasal vaccination, the mean antibody levels of IgG showed significant ($p<0.05$) increase in EPS-MhA2 and *M.* MhA2, while insignificant ($p>0.05$) gradual increase was observed in EPS-only and PBS groups before the mean antibody level started to decline at Week 2 and 3 post-vaccination. After that, the 2nd intranasal vaccination of EPS-MhA2 and MhA2 vaccine group showed that it had successfully induced IgG production. However, EPS-MhA2 vaccine group produced highest and significant ($p<0.05$) increase when compared with MhA2 vaccine group and the unvaccinated control groups at Week 5. Meanwhile for IgA, similar pattern with IgG was observed. At Week 1 post intranasal vaccination all groups except PBS showed gradual increase in antibody, but the levels were not significant ($p>0.05$). The booster dose showed again that EPS-MhA2 and MhA2 vaccine group significantly ($p<0.05$) increased the antibody levels of IgA at Week 5 as compared to the control groups. Among vaccinated groups, IgA in EPS-MhA2 vaccine group was still significantly ($p<0.05$) higher than MhA2 vaccine group.

Overall, from the development pattern of IgM, IgG and IgA, only EPS-MhA2 group consistently demonstrated a significant increased ($p<0.05$) in the production of IgM, IgG and IgA, especially after administration of booster doses at two weeks interval. The level of IgM started to increase after the 2nd booster on Week 4 and peaked at Week 6, while IgG and IgA reach their peak at Week 5, respectively when compared with the rest of the non-adjuvant groups.

4. Discussion

Chlorella sp. is known to produce and excrete large amounts of EPS in their extracellular environment (Delattre, Pierre, Laroche, & Michaud, 2016). *Chlorella* sp. culture medium was used to extract EPS. The yield EPS obtained in this study from *Chlorella* sp. culture using 10^6 CFU/mL was about 120 mg/L. In another study, yield of EPS obtained from *Chlorella* sp. was 174 mg/L (Xiao & Zheng, 2016), however, the study didn't report the concentration of the *Chlorella* sp. Physiochemical, structural, and biological activity of the EPS were determined to assess their biodegradability and toxicity to be used as vaccine adjuvant (Ahmed *et al.*, 2013). EPS produced in this study was in the form of white powder and was highly water soluble. The EPS showed a single symmetrical peak in the gel permeation chromatography (GPC) profile, which indicated that the extracted EPS was a homogeneous polysaccharide (Du, Yang, Bian, & Xu, 2017). The average molecular weight (Mw) of purified EPS was 2,398 Da, which was relatively small in size and categorized as low molecular weight. Xiao and Zheng (2016) suggested that EPS from *Chlorella* sp. should have high molecular weight due to its structural polysaccharides, which mainly consist of 1-6 galactose linkage. Noor-Hidayah (2014) also reported low molecular weight of *Chlorella* sp. to be as low as 2,274 Da. The low molecular weight EPS could be due to degradation of polysaccharide chain (Ziadi *et al.*, 2018) or due to limited number of monosaccharides present such as glucose, xylose, glucuronic acid and galactose (Raposo, de Morais, and de Morais, 2015; Xiao & Zheng, 2016). However, low molecular weight is advantageous as it associated with higher water solubility, stability and organism absorption (Ramnani *et al.*, 2012), hence, suitable to be used as adjuvant vaccines. Fourier-transform infrared spectroscopy (FTIR) spectra of *Chlorella* sp. EPS was in agreement with previous studies (Bayona & Garcés, 2014; Sajna *et al.*, 2013; Wang, 2011). The OH group at 3350.35 cm^{-1} could allow EPS to be absorbed easily by the mucus of nasal cavity and increase the nasal residence time for the vaccine (Sahoo, Chakraborti, & Mishra, 2011; Yang, Zhao, & Fang, 2008; Zaman *et al.*, 2013).

SEM properties of *Chlorella* sp.'s EPS were similar to the properties of EPS reported in previous studies (Ahmed *et al.*, 2013; Du *et al.*, 2017; Piermaria, Pinotti, Garcia, & Abraham, 2009) where the surface of EPS was dull and had pores. The microstructure of the produced EPS was similar to materials used to make plasticized films which indicated high water solubility, water absorbency and biodegradability. The EPS was also homogeneous matrix, which indicated the structural integrity and ability to make a film (Ahmed *et al.*, 2013). The morphology of EPS changed when it was mixed with sterile PBS excipient and the seed of formalin-killed whole organism MhA2 (Figure 6). Variations in EPS structure are common due to differences in physicochemical properties of the polysaccharide itself (Kanamarlapudi & Muddada, 2017). *Chlorella* sp.'s EPS did not show any cytotoxic effect on RAW 264.7 macrophage cell by MTT assay at all the tested concentrations between 1.56 and 100 µg/ml. This finding is consistent with previous study where the polysaccharide extracted from green microalgae *Haematococcus lacustris* on RAW 264.7 macrophage cells had immune-modulating activity without any detectable level of cytotoxicity (Park *et al.*, 2011).

Chlorella sp. EPS potential has been used as an adjuvant for *M. haemolytica* A2 (MhA2) vaccine at mucosal surfaces. The development patterns of the antibody in the blood serum are direct indicator of the humoral immune responses and generally used for examining the effectiveness of vaccine (Yang *et al.*, 2015). In this study, IgM, IgG and IgA production was monitor in the blood serum of Sprague Dawley (Figure 7). Generally, EPS-MhA2 adjuvant vaccine group showed higher level of antibodies, especially after 2nd intranasal vaccination at Week 2. Significant increase in IgM titers in EPS-MhA2 treated group starting at Week 4 until Week 6 could be due to opsonizing effect of serum IgM towards the exposure of EPS-MhA2. This result was supported by the fact that the IgM would be secreted out into the blood as the first antibody production to take action during the primary and secondary responses of any antigen (Black & Black, 2008). Then, the antibody response was followed by IgG production. In this study, there was increase of IgG level in Sprague Dawley in the two vaccinated groups (EPS-MhA2 and MhA2). The increase of IgG level from these groups was gradually stimulated starting at Week 4 and then became significantly high at Week 5. However, EPS-MhA2 vaccine showed significantly higher IgG responses compared to

MhA2 only vaccine. This observation indicated that the EPS as adjuvant had a good ability to maintain the *M. haemolytica* A2 vaccine in mucosal region of the Sprague Dawley, thus gave appropriate time for these antigens to stimulate memory cells during primary response. Due to the adequate production of these memory cells, the administration of the booster dose invigorated the significantly high level of IgG on systemic immune compartment. This finding was similar to the previous study where EPS adjuvant from *Aphanothece halophytica* (EPSAH) significantly enhanced serum OVA-specific total IgG and IgG2a production in immunized mice at two weeks post-immunization (Lei *et al.*, 2016). EPS-MhA2 vaccine also significantly increased IgA titer in the blood serum of Sprague Dawley at Week 5, especially after booster dose (Week 2) in EPS-MhA2 group compared to the other groups before the production of IgA declined on Week 6. The increase in IgA production in this study was consistent with the observations of IgA responses on mice that were vaccinated with the chitosan polysaccharide-adjuvanted vaccine (Svindland *et al.*, 2012).

The findings from this study suggest that the mechanism of the adjuvant is that the microencapsulation of the seeds will protect the seed from being phagocytized by the cells of the innate immune system. Neutrophils will engulf the adjuvant and degrade them, eventually exposing the seeds. Macrophages will clear the remaining EPS capsules and after a few hours, the process continues with the bindings of B cells receptors with the naked antigen seeds before the proliferation or cloning process of antibodies took place (Sompayrac, 2019). The receptors will bind directly to the antigens and continue the cloning process to produce huge quantities of specific antibodies into the tissues and blood. Instead of two weeks interval, it is better to give the booster at three weeks interval due to get more impactful immune responses due to the delay from the innate immune system to remove the EPS.

5. Conclusions

EPS from *Chlorella* sp. was found to possess suitable physiochemical properties to be a good adjuvant for intranasal delivery. EPS from *Chlorella* sp. significantly increase of IgM, IgG and IgA humoral immune responses and hence an effective adjuvant for *M. haemolytica* A2 vaccine. EPS as intranasal adjuvant enablabled *M. haemolytica* A2 vaccine seed to stay longer on the nasal mucosal epithelium. This will aid antigens to get better access to the lymphoid tissue and thereby stimulate an appropriate immune response in preventing the establishment of this particular infection. EPS obtained from *Chlorella* sp. could also be used as an adjuvant in other vaccines, especially those administered through intranasal or mucosal areas.

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