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## EVALUATION OF THE IMMUNOGENICITY OF EACH OF L-AMINO OXIDASE- AND L-ASCORBIC ACID-INACTIVATED HEPATITIS A VIRUS IN MICE AS POTENTIAL VACCINE CANDIDATES

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ABSTRACT

Hepatitis A virus (HAV) is one of the most common causes of acute viral hepatitis worldwide. Formaldehyde is the currently used inactivating agent in HAV vaccine processing despite of its adverse effects. The current study aimed to evaluate both L-amino acid oxidase (LAO) and L-ascorbic acid (LAA) as alternative inactivants for HAV and the immunogenicity of inactivated HAV in mice. Vero cell line was used for cultivation of HAV. The cytotoxicity of LAO and LAA on Vero cells was evaluated using 3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide (MTT) assay. The immunogenicity of each LAO- and LAA-inactivated HAV was examined in parallel with reference HAV vaccine in mice. Humoral (total IgG) and cellular immune responses (IFN- $\gamma$  and IL-5) were evaluated in mice sera using ELISA. Both LAO and LAA could efficiently inactivate HAV within 30 and 36 hrs post treatment, respectively, at concentrations of 0.4  $\mu\text{g}/\text{ml}$  of LAO and 1.5  $\text{mg}/\text{ml}$  of LAA. Inactivated vaccines were immunogenic to mice on both the humoral and cellular levels. LAO prepared vaccines showed a more promising immune reactivity than LAA prepared ones and alum-adsorbed vaccines were more immunogenic than non-adjuvanted ones. In conclusion, data recorded suggest that both LAO and LAA can be used as inactivating agents for HAV grown in cell culture. LAA- and LAO-inactivated HAV can be potential vaccines as they provide effective humoral and cellular immune responses comparable to that of the reference vaccine. The stability of test vaccines is recommended to be traced at different thermal conditions, in addition to different stabilizers and different pharmaceutical formulations must be tested trying to produce a lyophilized formula for long-term stability.

**Keywords:** Hepatitis A, vaccine, virus inactivation, L-amino oxidase, L-ascorbic acid, alum

INTRODUCTION

Hepatitis A virus (HAV) is the most common cause of infectious viral hepatitis which is acquired via the faecal-oral route. Hepatitis A is a common infection worldwide that is associated with unsafe drinking water, inadequate sanitation and poor personal hygiene (Wu and Guo, 2013). It is estimated that approximately 1.5 million clinical cases of HAV infection occur worldwide per annum. In addition, HAV infection is very common in underdeveloped countries such as Africa, parts of South America, the Middle East and India. In high endemic areas, hepatitis A occurs early in childhood; however, the development in sanitary conditions has resulted in a shift of the age groups affected by hepatitis A with increasing incidence in older age groups (Sartori et al., 2012). In Egypt, a study that has been performed in 2008 revealed that the frequency of HAV infection in children from low social class was high (81 %), while the prevalence rate was low (27.3 %) in those of higher classes (Franco et al., 2012). The disease is self-limited however HAV infection may result in acute liver failure and death, while risk increases with age and the presence of chronic liver disease (Wu and Guo, 2013). Prevention of HAV infection could be achieved by avoidance of exposure to contaminated food and water, proper disposal of excreta as well as administration of vaccine to those risk groups, such as health care workers (Zuckerman et al., 2009). Indeed, universal immunization would successfully control hepatitis A, although high costs and limited availability of vaccines preclude such a recommendation (Tahaei et al., 2012).

Vaccines against viral infections consist mostly of live attenuated or inactivated viruses (Stauffer et al., 2006). In 1992, two HAV formalin-inactivated vaccines, namely Havrix (GSK) and VAQTA (Merck), were available in United States and some other developed countries. These vaccines were developed by growing the virus in human diploid cell lines and then inactivated by treatment with formaldehyde (Karayiannis et al., 2004). Other formalin-inactivated HAV vaccines include Avaxim (Sanofi Pasteur) and Epaxal (Crucell Switzerland)

(Glück et al., 1992; Vidor et al., 1996). These vaccines are administered intramuscularly as a two-dose regimen, given at zero and six to 12 months. They generally well tolerated, with occasional reports of mild local reactions or, more rarely, fever and malaise (Karayiannis et al., 2004; Tahaei et al., 2012). Immunity induced by these vaccines is achieved in approximately 100 % of immunocompetent patients one month after receiving the recommended two doses (Fiore et al., 2006).

Several inactivating agents have been described to successfully inactivate viruses for vaccine purposes. Still, formaldehyde is the most widely used inactivating agent in vaccine industry for decades (Madhusudana et al. 2004). However, formaldehyde inactivation efficacy varies between vaccines concerning formalin concentration, time of inactivation and temperature. Generally, the higher the formalin concentration and temperature the faster is the inactivation, although this may adversely affect the antigenicity owing to thermal degradation and destruction of important epitopes (Sanders et al., 2015). As the integrity of the immunological epitopes of inactivated vaccines is virus-inactivant related. Therefore, it is a matter of interest to evaluate cheap and easily available alternative chemicals for fast and efficient inactivation of viruses without affecting its antigenicity (Madhusudana et al., 2004). Some studies have revealed that L-ascorbic acid (LAA) or vitamin C can be used as an inactivating agent for both DNA and RNA viruses while retained good antigenicity (White et al., 1986; Rawal et al., 1995; Madhusudana et al., 2004; Abd El-Razek et al., 2011). In addition, several studies reported that L-amino acid oxidase (LAO) present in animal secretory fluids, scorpion and snake venoms have strong antimicrobial activities against various pathogenic bacteria and viruses (Meenakshisundaram et al., 2009; Alyan et al., 2014; Kasai et al., 2015). Izidoro (2014) reported the possible inhibition of HIV-1 replication by LAO isolated from *Trimeresurus stejnegeri* venom. Based on the available information on virus inactivation efficacy of both LAO and LAA, the objective

of our study was to evaluate them as alternative inactivants to HAV and related immunogenic efficacy of both LAO- and LAA-inactivated HAV in mice.

## MATERIAL AND METHODS

### Hepatitis A virus strain and cell line

HAV strain HM175 was kindly supplied from water pollution department, National Research Centre, Cairo, Egypt. African green monkey kidney cell line [Vero cells, clone CCL-81] was kindly provided by Research and Development Sector, the Egyptian Holding Company for Biological Product and Vaccines (VACSERA, Giza, Egypt).

### Maintenance of Vero cell line

The 199 Eagle [E-199] medium (GIBCO, USA) supplemented with 200 mM L-glutamine, 10 % foetal calf serum (FCS) (Sigma Aldrich, USA), 100 IU/ml of penicillin and 100 mg/ml of streptomycin (Invitrogen, USA) was used to maintain Vero cell line in tissue culture flasks (TPP, Switzerland) according to **Doyle and Bryan (1998)** and **Mather and Roberts (1998)**, where the growth medium of the mother bottle was decanted. It was incubated at 37°C in 5 % CO<sub>2</sub> incubator (Jouan, France) until monolayer was developed. The monolayer was washed gently using sterile phosphate buffered saline [PBS] of pH 7.2. PBS was decanted and the cell monolayer was washed with 10 ml pre-warmed 0.25 % (W/V) trypsin-EDTA solution [Invitrogen, USA] and left in contact with cells for 15 - 30 seconds. Trypsin was decanted, the TC flasks were incubated with the trace trypsin at 37°C until the cells detach from the surface. Cells were dispensed in number of flasks to maintain cell count of  $\sim 2 \times 10^5$ /ml. TC flasks were kept till monolayers were developed. The actual cell number in the suspension was calculated by counting the cells using the haemocytometer and trypan blue dye (Sigma Aldrich, USA) exclusion method.

### Virus seed stock preparation.

Maintained Vero cells were inoculated with the HAV. The growth medium was carefully decanted and HAV as 0.1 MOI (multiplicity of infection) was inoculated onto Vero cells. HAV infected flasks were shaken for 15 minutes intervals to assure well virus distribution. Maintenance medium (100 ml) was then added to each infected flask. Infected flasks were incubated at 37°C and examined microscopically every day till the development of cytopathic effect (CPE). Flasks developed CPE were subjected to freezing and thawing three times to extract both cell free and cell associated virus from cells (**EL-Karamany, 1987**).

### HAV seed stock infectivity titration

HAV harvest was titrated on Vero cells, where HAV seed was 10-fold serially diluted using sterile E-199 medium. Dilutions of HAV were dispensed to Vero cells pre-cultured 96-well plates as 0.1 ml/well. Plates were incubated at 37°C for 7 days with daily microscopic examination using inverted microscope (Hund, Germany). Infectivity titre was determined according to **Reed and Muench (1938)** equation:

$$\text{PD Index} = (A-50\%) / (A-B) \times \text{Log dilution (10)}$$

Where A is the percentage of CPE at dilution immediately above 50 % and B is the percentage of CPE at dilution immediately below 50 %. After that index was applied to the dilution that produced the percentage of cytopathic effects immediately above 50 %.

### Chemical inactivants

L-amino oxidase (LAO) and L-ascorbic acid (LAA), used for HAV inactivation in this study, were purchased from Sigma Aldrich, USA. They were prepared at a concentration of 1 mg/ml and processed for evaluation of their safe concentrations. LAA solution contained CuSO<sub>4</sub> at a final concentration of 5 µg/ml.

### MTT assay

Cytotoxic effects of both LAO and LAA was determined on Vero cells using 3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide (MTT), where Vero cells were dispensed in 96 well plates. Plates were incubated till confluency. Test inactivants were 2-fold serially diluted and 24 hrs post incubation at 37°C, dead cells were washed out using PBS. Remaining viable cells were stained with MTT stain as 50 µl (5 mg/ml)/well. Plates were incubated at 37°C for 3-4 hrs. Developed crystals were dissolved using 0.4 % acidified iso-propanol or Dimethyl sulphoxid (DMSO). Developed colour was read at 570 nm wave length using Biotek- ELX-800 ELISA microtiter plate reader. The viable cell number was calculated according the equation: Viable % = OD Test × 100 / OD of cont. The safe concentration of test inactivant was determined.

## Determination of inactivation kinetics of HAV

Inactivation kinetic relative to time post treatment with LAO and LAA was determined according to **Madhusudana (2004)** and **El-Karamany (1987)** where 1 ml of chemically treated virus was collected at time interval of one hour. Virus samples were 10-fold serially diluted 10<sup>1</sup> - 10<sup>8</sup> in 199-E medium. Prepared dilutions were dispensed onto 24 hrs pre-cultured Vero cells in 96 well plates (TPP-Swiss). Infected plates were kept at 37°C in 5 % CO<sub>2</sub> (Jouan-France) with daily microscopic observation using inverted microscope for detection of the CPE. The 50 % end point induced CPE was determined according to **Reed and Muench (1938)**.

### Acute toxicity of test inactivants

Acute toxicity of each test inactivants was performed according to **Abd El-Razek et al. (2011)**. Intraperitoneal acute toxicity was studied in Swiss Webster male mice. The animals had free access to feed and drinking water. Mice were allocated into groups (10/cage). Test chemicals safe concentrations were administered intraperitoneally. General symptoms of toxicity and mortality were observed for 24 hrs, after which the animals were left for further 7 days for delayed toxicity.

### Aluminum phosphate (Alum) adjuvant

The solutions of each of 0.63 M AlCl<sub>3</sub>.6H<sub>2</sub>O and 0.3 M Na<sub>3</sub>PO<sub>4</sub>.12H<sub>2</sub>O were prepared in 40 ml normal saline. Prepared solutions were 0.2 µm filtered. Contents were stirred continuously during the procedure at 40 to 60 rpm. After wards, 0.3 M Na<sub>3</sub>PO<sub>4</sub>.12H<sub>2</sub>O solution was added to the mixing bottle, and then 300 ml normal saline was added. The antigen was also added followed by addition of 0.63 M AlCl<sub>3</sub>.6H<sub>2</sub>O solution to the mixing bottle. The pH was maintained between 6.5 - 6.8. The final volume was adjusted with sterile normal saline and the suspension was mixed for 2 hrs at 37°C (**Gupta, 1998; Lindblad, 2004**).

### Mice immunization

Six to eight weeks old Swiss Webster male mice, housed at the animal facility of VACSERA, Giza, Egypt, were used in this study according to the recommendations of Animal Care and Use Committee (ACUC). Alum was used as adjuvant at concentration of 0.35 mg/ml to enhance the immune response to the injected inactivated virus. In this study, five groups of mice, 10 each, were immunized subcutaneously. Four groups were immunised individually with LAO- and LAA-inactivated HAV, alum adsorbed and none-adsorbed, while the fifth group was immunised with the currently marketed HAV vaccine Havrix. Havrix is an alum-adsorbed commercial vaccine (Glaxo SmithKline) containing 1440 ELISA units [EU] of formalin-inactivated HAV as a positive control. Immunized mice were bled through retro orbital plexus. Immune sera prepared from blood samples collected from each group at a two-week interval post-immunization. Antibody level [IgG] and cytokines [IFN-γ and IL-5] produced post-immunization were monitored.

### Detection of HAV-specific antibodies in mice sera

Antibodies against HAV vaccine were detected in post-immunization sera samples using enzyme-linked immunosorbent assay (ELISA) according to **Abd El-Razek et al. (2011)**, where sera samples were diluted as 1/100 in dilution buffer (PBS + 1 % BSA) and added to HAV antigen pre-coated 96-well maxisorb ELISA plates. Sera samples were serially diluted and plates were incubated for an hour at 37°C. Plates were washed three times with 300 µl of 1× wash buffer (PBS + 0.05 % Tween 20) using automated ELISA plate washer for better washing performance. Then, 100 µl of anti-mouse IgG-HRP conjugate were added to all wells leaving one empty for the substrate blank. Plates were mixed gently for 5-10 seconds and then incubated for 60 minutes at 37°C. Plates were washed as previous. Tri Methyl Benzidine (TMB) substrate buffer (Sigma Aldrich, USA) was added as 100 µl/well and plates were kept in dark for 20 minutes at room temp. The reaction was stopped using 100 µl of 2N H<sub>2</sub>SO<sub>4</sub>. Optical density of developed colour was measured at 450 nm using BioTek-XL-800-USA) ELISA reader within 60 min.

### Cytokines determination

The amounts of IFN-γ and IL-5 in mice immune sera were assayed using specific sandwich ELISA kit for each cytokine (eBiosciences, USA) according to the manufacturers' instructions.

### Statistical analysis

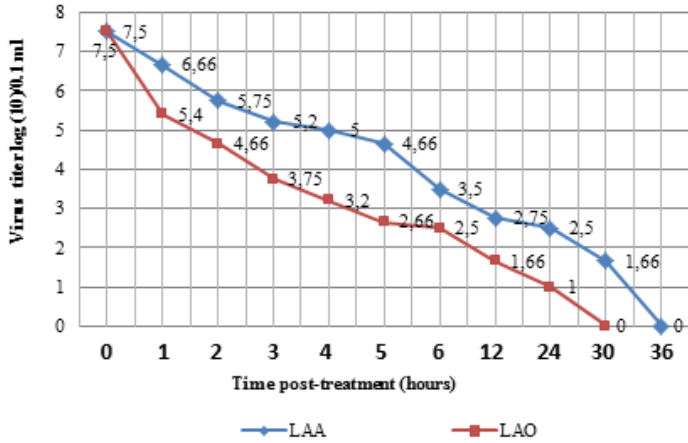
Results are expressed as mean values for three three independent experiments. Comparison between the different groups was made using unpaired student t-

tests to assess significance using GraphPad Prism 5 software. Differences at *P* values less than 0.05 were considered significant.

**RESULTS AND DISCUSSION**

**Treatment with LAA or LAO could successfully inactivate HAV**

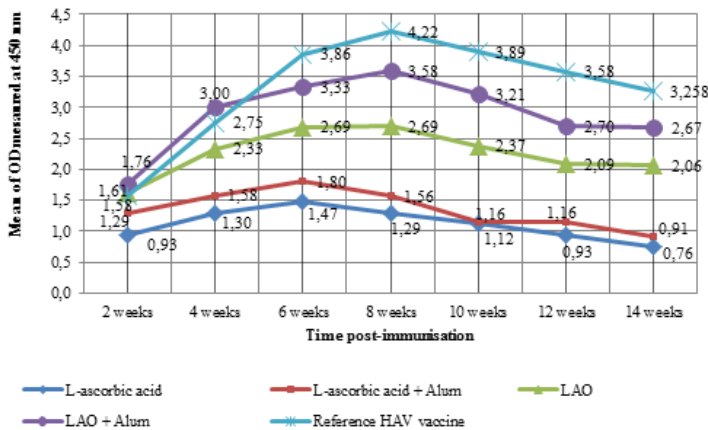
Testing the cytotoxic effects of test inactivants LAO and LAA showed that the safe concentrations were 0.4 µg/ml and 1.5 mg/ml, respectively. These concentrations were used to inactivate HAV, which showed no toxicity to mice as all inoculated mice were alive throughout the designed 7-day period of test. Data recorded revealed that HAV was completely inactivated within 30 hrs and 36 hrs post-treatment with LAO and LAA, respectively, and no residual infective virus was detected (Figure 1).



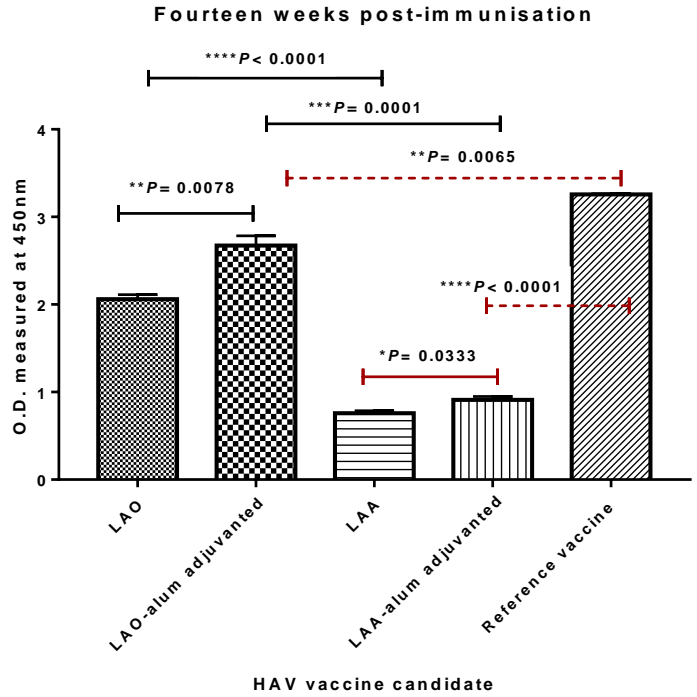
**Figure 1** Evaluation of inactivation kinetics of HAV post-treatment with each LAO and LAA relative to time using cell culture assay.

**Each of LAA- and LAO-inactivated HAV induced humoral and cellular immune responses in mice**

Regarding the humoral immune response, total HAV-specific IgG response was monitored. Data recorded revealed that HAV-specific IgG antibodies were detectable in the mice immune sera as early as two weeks and the peak was detected at the 6<sup>th</sup> week and 8<sup>th</sup> week post-immunization with test LAO-inactivated vaccines either alum adjuvanted or non-adjuvanted and LAA-inactivated vaccines either alum adjuvanted or non-adjuvanted, respectively. There was a significant difference (*P* < 0.05) in the level of the antibody production, which was detected 14 weeks post-immunisation with LAO prepared vaccine than those detected post-immunization with LAA prepared vaccines. Alum adjuvanted vaccines showed a significant elevated antibody level (*P* < 0.05) than non-adjuvanted ones throughout the immunization course. Based on the antigen content, the purchased reference vaccine showed the highest level of antibody response (Figures 2, 3).

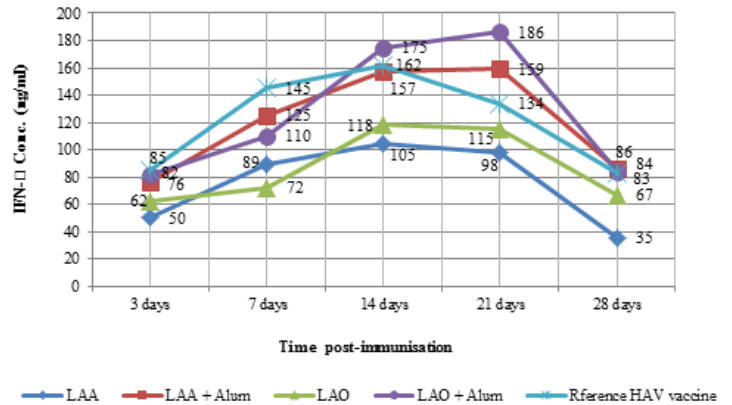


**Figure 2** IgG immune response of mice immunised with alum adjuvanted and non-adjuvanted LAA- and LAO-inactivated HAV vaccines, relative to time post-immunisation.



**Figure 3** IgG immune response in mice 14 weeks post-immunization with HAV vaccines. Data presented as mean ± SE of three replicates.

Concerning the cellular immune response, IFN-γ and IL-5 cytokines were estimated in immunised mice sera. The production of these cytokines could be detected as early as 3 days post-immunisation with HAV-inactivated vaccines. The level of IFN-γ was significantly higher (*P* < 0.05) in case of both alum adjuvanted LAA- and LAO-inactivated HAV vaccines than non-adjuvanted ones until 28 days post-immunisation. The alum adjuvanted LAO- and LAA-inactivated vaccines showed high levels than that produced by purchased positive control vaccine (*P* < 0.05) at 14 days and 21 days post-immunisation. At 28 days post-immunisation, there was no significant difference (*P* < 0.05) between the level produced by LAO-adjuvanted vaccine and that of the purchased vaccine (*P* > 0.05), while that of LAA-adjuvanted vaccine was slightly higher at *P* < 0.05 indicated by one asterisk. LAO-inactivated vaccines produced significantly higher levels of IFN-γ (*P* < 0.05) than that produced by LAA-inactivated ones at 14, 21 and 28 days post-immunisation denoted by four asterisks, except at 28 days, there was no significant difference (*P* < 0.05) between adjuvanted ones (Figures 4, 6).



**Figure 4** Estimation of IFN-γ concentration in mice sera post-immunisation with LAO- and LAA-inactivated HAV vaccines using specific sandwich ELISA.

IL-5 level was significantly higher (*P* < 0.05) in both LAO- and LAA-inactivated vaccines adjuvanted with alum than those non-adjuvanted ones at 14, 21 and 28 days post-immunisation. LAO-inactivated vaccines produced significantly higher levels of IL-5 (*P* < 0.05) than that produced by LAA-inactivated ones. The IL-5 level produced by purchased vaccine was significantly higher (*P* < 0.05) than that produced by test vaccines at 14, 21 and 28 days post-immunization except LAO-adjuvanted one which was significantly higher (*P* < 0.05) than control vaccine at 21 days (Figures 5, 6).

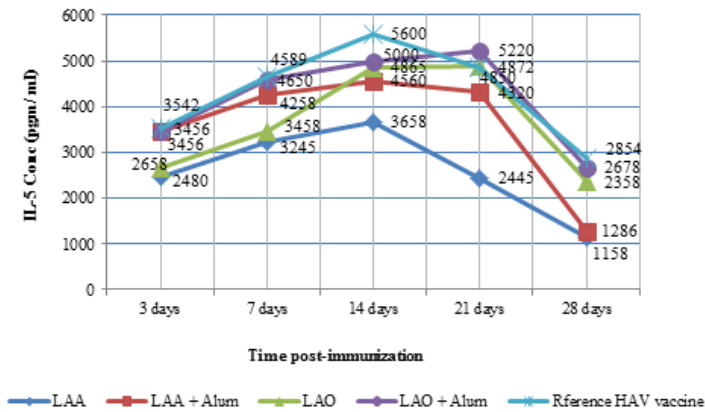


Figure 5 Estimation of IL-5 concentration in mice sera post-immunization with LAO- and LAA-inactivated HAV vaccines using specific sandwich ELISA.

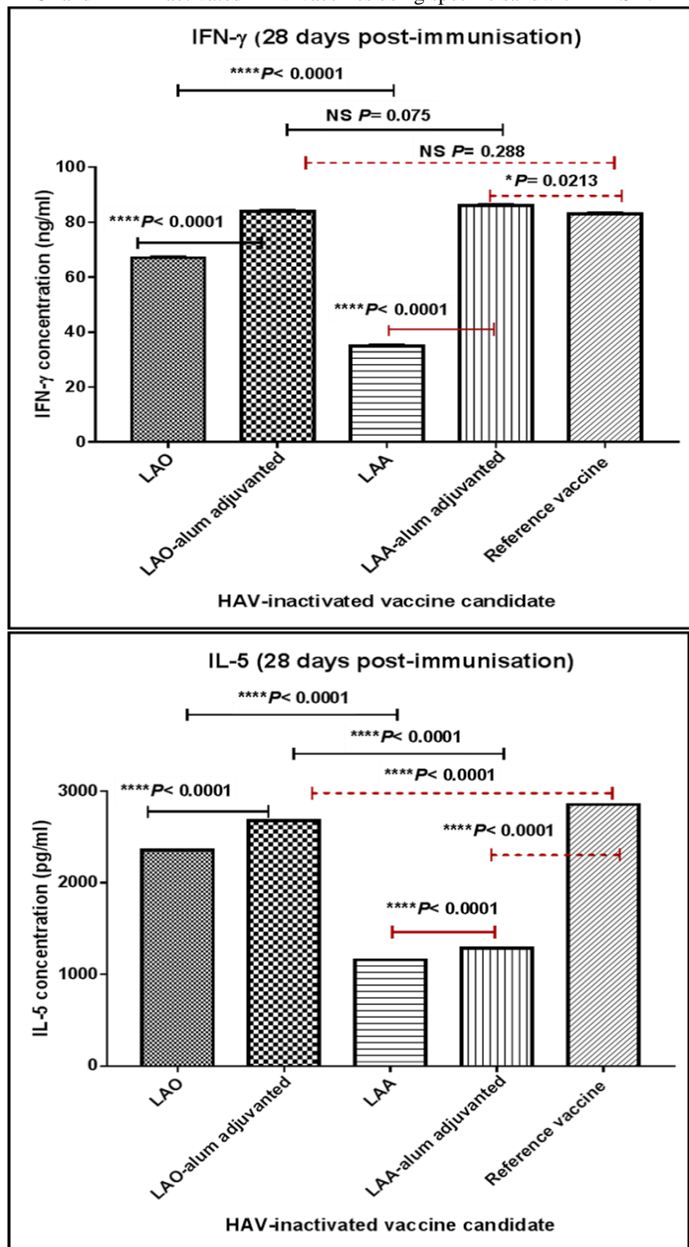


Figure 6 IFN-γ and IL-5 concentrations in mice sera at 28 days post-immunization with LAO- and LAA-inactivated vaccines.

In the past two decades, the HAV cases have declined in several parts of the world due to partly the developed vaccines (Kanyenda et al., 2015). The commercially available HAV vaccines contain formalin-inactivated HAV, grown on human cell lines and purified, that has been adsorbed with alum to enhance its immunogenicity. While they are considered efficacious, often multiple boosters are required to confer protection against HAV. These vaccines are also very

expensive to produce, available in limited quantities, and must be kept cold until they are administered by injection (Mitchell and Galun, 2003). Still in developing countries, such as Egypt, HAV vaccination may be considered on the basis of epidemiological and cost-effectiveness concerns. Thus, the available HAV vaccines are not yet commonly used (Franco et al., 2012). Viral inactivation is an important procedure in vaccine development against viral infections (Stauffer et al., 2007). However, the inactivating agent may have an effect on the viral epitopes pattern to which the antigenicity and/or immunogenicity of vaccine is related (Blackburn and Besselaar, 1991). Thus, the inactivation procedures may drastically impair induction of neutralizing IgG antibodies responses for most viruses (Bachman et al., 1994). Formaldehyde is the inactivating agent used for HAV vaccines (Chowdhury et al., 2015). However, it was observed that for formalin inactivation concentration, pH, temperature and medium composition are extremely critical factors. Formalin was found to require at least 24 – 96 hrs at 4°C –37°C for inactivation. In addition, the higher concentrations of formaldehyde and temperature used to speed up the inactivation may harmfully affect the immunogenicity of inactivated virus (Chowdhury et al., 2015; Sanders et al., 2015). Seriously, an incomplete formaldehyde inactivation procedure may be fatal for public health. For example, incomplete inactivation of the Venezuelan equine encephalitis vaccines, prepared by formalin inactivation, was the cause of the outbreak of the disease during the 1969 – 1972 pandemic in Central America (Brown, 1992). Furthermore, formaldehyde which is an alkylating agent inactivates viruses via chemical reaction with viral capsid proteins and nucleic acid (Budowsky et al., 1991), and it has been classified by International Agency for Research in Cancer (IARC) under group 2A. However, there is no epidemiological data referring to the carcinogenic risk of the alkylating agents on humans (Chowdhury et al., 2015). Owing to previous data, there is a need for available, cheap and efficient alternatives for fast inactivation of HAV without affecting its immunogenicity allowing HAV vaccines to be less expensive to be manufactured and more amenable to mass vaccination programs. Thus, the main objective of the present study was to evaluate the efficiency of LAO and LAA as inactivants of HAV and examine the immunogenicity of each LAA- and LAO-inactivated HAV compared with the currently available formaldehyde prepared vaccine.

In the present study, both test inactivants could successfully inactivate HAV using safe concentrations (0.4 µg/ml of LAO and 1.5 mg/ml of LAA), within 30 hours and 36 hours post-treatment, respectively. LAO has previously showed remarkable virucidal activities to viruses such as varicella-zoster virus and herpes simplex virus type 2 (Zedan et al., 2003; Alyan et al., 2014). In addition, data recorded of HAV inactivation by LAO in this study is consistent with results of Aly Mohamed and his colleagues (unpublished data), despite the use of another virus model namely Rift Valley fever virus that was completely inactivated with LAO within 6 hrs, and the immune potential of this vaccine was better than that of the known β-propiolactone (BPL)-inactivated vaccine. Recently, snake venom LAO has been recognized as a multifunctional protein with promising biomedical applications because of its antimicrobial, anti-HIV, anticoagulant, and inducing of platelet aggregation. LAO enzyme is acting specifically on L-amino acids and generally on hydrophobic amino acids (Du et al., 2002). The inactivation of HAV by LAO is possibly due to the oxidizing stress of LAO attributed to the ability of the enzyme to localize H<sub>2</sub>O<sub>2</sub> to the target cells through channels in its structure that would direct the H<sub>2</sub>O<sub>2</sub> product to the exterior surface of the protein, near the glycan moiety. Thus, the glycan moiety is thought to be involved with LAO-target cell interaction. Accordingly, the virucidal activity of LAO was the base on which it was used as virucidal agent to prepare an improved HAV vaccine (Shebl et al., 2012).

Regarding the cytotoxicity of LAO, our findings were found to be in agreement with Shebl et al. (2012) study which revealed that LAO showed significant cytotoxicity to Vero normal cells at concentrations higher than 0.4 µg/ml. The LAO cytotoxicity may be attributed to the released H<sub>2</sub>O<sub>2</sub> enhancing the oxidative stress on cell nucleus causing cell death and progressive apoptosis. Concerning the use of LAA as an inactivant for HAV, our findings are supported by Madhusudana et al. (2004) that ascorbic acid can be used as an inactivating agent for viruses grown in cell line although the authors used it for rabies virus. In addition, earlier experiments used LAA as an inactivating agent for number of viruses including vaccinia virus (Turner et al., 1964) HIV virus (Rawal et al., 1995) and *in vitro* infectivity of herpes viruses and paramyxoviruses (White et al., 1986). Abd El-Razek et al. (2011) study revealed also the complete effective inactivation of Rift Valley fever virus using LAA for vaccine development. The mechanism whereby ascorbic acid inactivates viruses is not fully understood. Although, it was partly explained by the presence of oxygen which is essential and ascorbic acid undergoing auto-oxidation results in the formation of OH groups that could bring about the inactivation of the cell free viruses (Murata et al., 1986).

The mice immunisation experiments performed in this study revealed that both inactivated HAV vaccines induced a good antibody (humoral) immune response as indicated by the results of the HAV-specific IgG ELISA. Regarding the immunogenicity of LAO-inactivated vaccine, the results revealed that HAV-specific antibodies were detected 14 days post-immunization and this was in agreement with Keefe et al. (1989), despite their trial was conducted on the

clinical level. Whilst, the immune response peak was on the 8<sup>th</sup> week post-immunization with LAO or LAO alum adjuvanted vaccine, similar to the antibody response produced by HAV reference vaccine, and on the 6<sup>th</sup> week post-immunization for LAA or LAA alum adjuvanted vaccine. Subsequently, the level of antibody production starts to decrease. Natural infection with Hepatitis A virus leads to life long detectable antibody in most individuals, whereas vaccine induced antibody levels wane over time. In healthy adults, vaccine induced anti-HAV has been observed to decrease rapidly from one month after the booster vaccination until six month later, followed by a rather constant decrease over the subsequent two years, approximately 14 % per year (Van Damme et al., 1994). Thus, in most countries, booster-vaccination policy is guided by manufacturers' recommendations, national authorities, or both. Data showed that after a full primary vaccination course, protective antibody amounts persist beyond 10 years in healthy individuals, and underlying immune memory provides protection far beyond the duration of anti-HAV antibodies (Van Damme et al., 2003).

One of the major cellular effectors is the CD4+ helper T cells that elaborate cytokines which enhance both antibody and cellular immune responses. For investigating the cellular immune response in immunised mice and the predominant phenotype, cell-mediated (Th1) or humoral (Th2) immune responses elicited by immunization with LAO- or LAA-inactivated HAV, the level of two cytokines IFN- $\gamma$  (Th1 cytokine) and IL-5 (Th2 cytokine) were estimated in the post-immunisation sera. The cytokine-specific sandwich ELISA results revealed that the mice immune response generated with both LAA- and LAO-inactivated HAV immunisation was a mixed Th1/Th2 immune response profile similar to that produced by HAV reference vaccine. However, the quantitative measurement of the level of each cytokine indicated that the concentrations of Th1 cytokine IFN- $\gamma$  (cellular immunity) was higher than that of Th2 cytokine IL-5 (humoral immunity). These results indicated that the extent of up-regulation of IFN- $\gamma$  production was the highest when compared to that of IL-5, confirming a significant Th1 immune response. Accordingly, there was distinct humoral and cellular immune responses post-immunisation with both LAA- and LAO-inactivated HAV. Consistent with our results, Schmidtke et al. (2005) study results which revealed that both distinct B and T cell responses were determined within 14 days after immunisation with formalin-inactivated vaccine (Havrix) in humans. However, the cellular immune response in the current study might be the predominant one which is fitting for vaccination against viral infections by HAV. That is because the cellular immune response is very vital for combating viral infections (Lappin and Campbell, 2000). In addition, clinical and experimental evidence proved the hypothesis that HAV hepatocellular damage and the efficient elimination of virus-infected hepatocytes are mediated by virus-specific, proliferating T lymphocytes derived interferon (Schmidtke et al., 2005). The data recorded in this study concerning type of immune responses was also in agreement with Cederna et al. (1999), despite their trial was on the clinical level, which revealed that HAV antibody and proliferative effective T cell response were elicited by a formalin-inactivated HAV vaccine in the immunized subjects. However, antibodies produced against HAV are present over an extended period in the human sera, indicating the likely importance in maintaining providing a long-term immunity (Wang et al., 1996). These results indicated the high and good immunogenicity of each of LAA- and LAO-inactivated HAV in Swiss Webster mouse strain. Although, there was no significant difference in the immunogenic potential between the LAO- and LAA-inactivated HAV; both showed equivalent antigenic potency as measured by indirect ELISA.

The concentrations of Th1 cytokine IFN- $\gamma$  in alum adsorbed LAA- and LAO-inactivated HAV was higher in those non-adsorbed ones. This finding is consistent with the practically established concept that alum adjuvant is administered with antigens in experimental murine and rabbit immunization studies to enhance immunity (Gupta, 1998; Lindblad, 2004). Indeed, all HAV commercially available formalin-inactivated vaccines are adsorbed onto alum as an adjuvant (Karayiannis et al., 2004; Tahaei et al., 2012). Previous published studies concerned the cellular immune response to vaccine prepared using LAO as a natural product is very rare as the majority of inactivating agents are chemicals in nature not natural derivatives. However, the use of LAA as an inactivating agent showed similar immune response, and particularly the cellular immune response pattern as in case of inactivation of rabies virus reported by Madhusudana et al. (2004) and Abd El-Razek et al. (2011). Thus, it was hardly to compare HAV vaccine potentials to other prepared vaccines using another inactivating agent but using the same technique except in case of preparing Rift Valley fever virus vaccine inactivated with both BPL and LAO despite the previous reporting of LAO as a virucidal agent to Rift Valley fever Virus, Vesicular Stomatitis Virus (VSV), Herpes and Adeno viruses (Abd El-Razek et al., 2011; Alyan et al., 2016). Accordingly, data recorded regarding the immune response is in agreement with one of the authors of this study, Aly Mohamed and his colleagues (unpublished data), that LAO-inactivated Rift Valley fever vaccine was high immunogenic than the BPL-inactivated one. The use of alum-adjuvanted vaccines has showed a better immune response than non-adjuvanted ones.

## CONCLUSION

It can be reported that both LAA and LAO are promising and equally efficient for inactivation of HAV. Each of LAA- and LAO-inactivated HAV appears to provide potentially effective anti-HAV vaccines owing to the finding that they induced both good humoral and cellular immune responses. The immune potentials of both LAA- and LAO-inactivated vaccines are almost equally to that of formalin-inactivated HAV vaccines currently available in the Egyptian market. There was no distinguished difference in the values of cellular immune response parameter of interest. Both HAV-inactivated vaccines were biologically of near bio-reactivity to immune system. Alum as adjuvant is useful to enhance the immune potentials of inactivated HAV on the experimental level. However, the present study is limited by showing the immune response only for days and/or weeks, thus long-term tracing of immune response on both the cellular and humoral levels is warranted. The HAV-inactivated vaccines in the current study were not challenged in experimental animals as human is the only reservoir host of HAV and the lack of animal model that mimics the human infection. An extensive investigation of the HAV vaccine-induced immune response should include the analysis of circulating HAV-specific T lymphocytes. As storage, handling and the heat stability of vaccines are consequently matters of great concern. Thus, studying the stability either real time or accelerated one and tracing the accumulative effect of residual LAO is recommended on the biochemical pathological level. Both LAO and LAA should be further compared with other inactivating agents along with testing different inactivation agents and/or vaccine adjuvants to maximize the immune response and formulate the best suitable method for HAV vaccine preparation.

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