ARTÍCULO ORIGINAL

Reactogenicity of *Bordetella pertussis* vaccines formulations using aluminum adjuvants in Sprague Dawley rats

Reactogenicidad de formulaciones de vacunas de *Bordetella pertussis* utilizando adyuvantes de aluminio en ratas Sprague Dawley

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Abstract. Combined vaccines for childhood are a strategy in the prevention of several diseases. These can maximize protection and decrease immunization schedules in children. New candidates are getting closer to being able to meet these needs, but they raise numerous strategic questions related to formulation and regulatory aspects. In addition to being immunogenic and protective must have low reactogenicity when combined with other antigens. Adjuvants are important components in achieving these combinations. Therefore, a reactogenicity study was designed for two *Bordetella pertussis* formulations containing hydroxide or aluminum phosphate in Sprague Dawley rats. Both formulations dose were administered in 0.2 mL intramuscularly. Clinical evaluations, body weight, water consumption, food, temperature, muscle volume, dermal irritability and pathological studies with special interest at the inoculation site were carried out. Only differences in body temperature and muscle volume were found with a slight increase in values with return to normal. The macroscopic study showed lesions at the site of inoculation, considered characteristics of aluminum adjuvants, such as granulomatous abscesses and the increase in regional lymph nodes near the inoculation site. As conclusion, there are no differences between the formulations of *B. pertussis* with hydroxide or aluminum phosphate resulted in low reactogenicity.

Keywords: Reactogenicity; *B. pertussis*; Adjuvants; Sprague Dawley rats

Resumen. Las vacunas combinadas resultan una estrategia importante en la obtención de vacunas múltiples para la infancia y el uso de adyuvantes es un componente de gran valor en lograr estas combinaciones, además de ser inmunogénicas y protectoras deben tener baja reactogenicidad, cuando se combinan con diferentes antígenos. Por esta razón, se diseñó un estudio de reactogenicidad a dos formulaciones que contenían hidróxido y fosfato de aluminio con antígenos de *Bordetella pertussis* en ratas Sprague Dawley. Se administró a cada grupo de ensayo una dosis correspondiente de ambas formulaciones en 0,2 mL por vía intramuscular. Se realizaron observaciones clínicas, comportamiento del peso corporal, consumo de agua, alimentos, temperatura corporal, volumen muscular, irritabilidad dérmica y estudios anatomopatológicos macroscópicos, con especial interés en el sitio de inoculación. No se observaron síntomas, ni muertes en los animales durante el estudio. Tampoco se encontraron diferencias entre los grupos experimentales en cuanto al peso corporal, el consumo de agua y de alimentos; los estudios de temperatura corporal y volumetría muscular evidenciaron un ligero incremento en los valores, los cuales involucionaron rápidamente a la normalidad. En el estudio anatomopatológico macroscópico se observaron lesiones a nivel del punto de inoculación, consideradas propias de los adyuvantes que contienen aluminio, tales como formaciones abscedadas de tipo granulomatosas y el aumento de los ganglios linfáticos regionales cercanos al punto de inoculación. Se concluye que las formulaciones en hidróxido y fosfato de aluminio con antígenos de *B.pertussis* resultaron ser de baja reactogenicidad.

Palabras clave: Reactogenicidad; *B.pertussis*; Adyuvantes; Ratas Sprague Dawley

Introduction

Obtaining multiple or combined vaccines has been a justified strategy, in addition to the possibility of reducing the number of immunizations to which children are subjected, mainly for the prevention of several diseases caused by different strains or serotypes of the same microorganism (FDA 1997; Walker and Dull 2017). There are several combined vaccines designed for the prevention of diseases such as Diphtheria, Tetanus and Pertussis (DPT vaccine), pentavalent (HeberPenta®-L),

against *Haemophilus* type b, Hepatitis B plus DPT, *Shigella* combined vaccine, against malaria and cancer, among others; this vaccines can have in their formulations new or known adjuvants (Bacardí *et al*. 2013; Silva *et al.* 2015; Pandey *et al.* 2016; Rampling *et al*. 2016).

Finlay Institute of Vaccines works in obtaining vaccines for the prevention of different diseases and one of its lines is combined vaccines for childhood. In this sense, *B. pertussis* (Bp) antigens are currently evaluated as one of the components of different combined vaccines. The component of inactivated cells of Bp in the current DPT and HeberPenta®-L vaccines of the Cuban immunization scheme, are adjuvanted in aluminum hydroxide ($Al(OH)_3$) and aluminum phosphate ($AlPO_4$) as well, similar to others kind of vaccines like VA-MENGOC-BC® as well mentioned before (Oliva *et al.* 2019). One of the critical elements for multivalent vaccines containing inactivated Bp cells is reactogenicity or local response at the immunization site (FDA 1997). Considering the possibility of combining Bp cells with other antigens that need to be adjuvanted in $AlPO_4$, a reactogenicity study was designed two formulations with this antigen adjuvanted to $Al(OH)_3$ or $AlPO_4$ in Sprague Dawley (SD) rats.

Materials and methods

Animals and husbandry

Male SD rats were purchased from CENPALAB, Havana Cuba (from Spanish: Centro Nacional para la Producción de Animales de Laboratorio) at an age of 7-8 weeks and were housed in Tecniplast® rat cages at the Animal Care Facility at Finlay Institute of Vaccine. Dimensions and model: 1354G Eurostandard Type IV, 595 x 380 x 200 mm, floor area 1820 cm, PEI plastic and BPA-Free. Rats were provided with specialized feed for rodents (ALYco®), and the water used was provided in acidified (2.5-2.7 pH) water bottles (750 mL volume). Both food and water were available *ad libitum*. The animal room was maintained at a temperature of 22 ± 2°C and a relative humidity of 55 ± 5%. These parameters were recorded daily in addition to maintaining 12-hour light and dark cycles. Rats were allowed to acclimatize to their surroundings for one week prior to the commencement of the experimental protocol and were randomly placed into groups of 10. All protocols were approved by the Animal Care Committee and the Biosafety Committee at Finlay Institute of Vaccine.

Formulation and vaccination protocol

The formulations were prepared (*Table 1*) from a batch of Bp (strains 165, 509 and 134) obtained in the production plant of the Finlay Institute of Vaccines. The adsorption process used for the combination of Bp (whole-cell inactivated) and $Al(OH)_3$ was similar to DPT vaccine. The Bp was absorbed in $AlPO_4$ with pH from 6.0 to 6.5 at room temperature, in presence of 0.9% NaCl as a buffer solution. Both formulations Bp were maintained at a concentration of 32 OU/mL, while concentration of aluminum (Al^+) was 0.7 mg/mL approximately for each adjuvant.

Study lasted 7 days; for which three treatment groups by randomization were formed, receiving a dose of the formulated vaccines under study (*Table 1*). Rats were immunized intramuscularly (IM) to use the same route used in humans, in the posterior middle region of the inner side of both legs (100 µL in each leg). This study was designed following the recommendations and guidelines issued by the WHO for the evaluation of vaccines (WHO 2013).

Table 1. Experimental design

Group	Sex	n	Formulation (AgC/AdC/vol.)	Dose & vol. to animals	n euthanasia (7 days)
1	Male	10	Control (PBS)		
2	Male	10	Bp/$AlPO_4$ (16OU/346µg/0.5mL)	6.4OU/200µL	10
3	Male	10	Bp/$Al(OH)_3$(16OU/346µg/0.5mL)	6.4OU/200µL	10
	Total	30			

Legend: n- Number of animals; PBS- Phosphate Buffer Solution; AgC- Antigen concentration; AdC- Adjuvant concentration; Vol- Volume of human dose; Bp- *B.pertussis*; $AlPO_4$ – Aluminum phosphate; $Al(OH)_3$ – Aluminum hydroxide; OU- Opacity Units.

Clinical observations and symptoms
All observations started from the experimental zero time (T0), which was considered as the same day of vaccination. Animals were monitored daily (three times a day during first 72 hours) paying special attention to the rats when evaluating their food and water intake, body weight, diameter of the leg muscles, skin and temperature.

Body weight, water and food consumption
At the time of inoculation, rats were weighed to determine their starting weight at the beginning of the study. All animals were identified by the ear punch-out method and weighed at week (end-point) to monitor their weight as a measure of toxicity. Water and food consumption were measured at the start of the vaccination protocol (T0) and alternating days thereafter; daily water and food consumption (mL or g/animal/day respectively) were calculated based on the amount of food and water consumed over the span of a week.

Body temperature and muscle diameter
The body temperature of the rats was measured with a mercury clinical thermometer thin (Hemeco, China) rectally. Body temperature was measured before the inoculation and 72 hours after (intervals of time of 4, 24, 48 and 72 hours).
The evaluation of the muscle diameter was performed as previously described for rats and mice (Oliva *et al.* 2019; Fraleigh *et al*. 2019) with a digital caliper (Mastercraft, Electronic Caliper with digital display, 6",150 mm) by measuring the diameter of the inoculated limb before the intramuscular vaccination at 4, 24, 48 and 72 hours after vaccination to assess local inflammation.

Dermal irritability
Dermal irritability test (DI) was performed in similar way and as previously described for rats (Oliva *et al.* 2019). The test is made by Draize method modified and as predictive element of local reactogenicity for clinical essays (Draize *et al.* 1944) and has been recommend by World Health Organization (WHO 2013). Score of DI was evaluated in same times previously described for temperature and muscle diameter.

Anatomopathological studies
The anatomopathological studies for gross necropsy were performed immediately after euthanasia at 7 days. All organs and sites (lymph nodes) of vaccine administration were examined macroscopically.

Statistical analysis
Statistical analyses were performed using Graph Pad Prism 5. Multiwise group analyses were performed using a nonparametric ANOVA with a Dunn's post-hoc test. The significance level was adjusted for multiple comparisons using Bonferroni test. Data were considered significant when $p \leq 0.05$.

Results
No mortality or abnormal clinical signs were noted during the study. All of the male SD rats increased their body weight during the 7 days of the study, no statistically significant difference was observed between groups (*Figure 1*). The weight increase curves of the rats were similar to those observed for this specie and in line with the growth curves available from Charles River and similar to other studies made by us with historical data from our animal's facility as well.

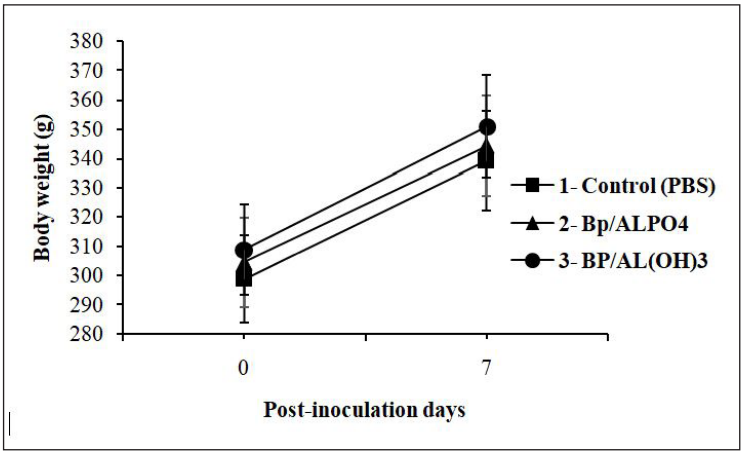

Figure 1. Body weight time course of rats with formulations adjuvants *B. pertussis* vaccine. Each value represents the average ± SEM of the 10 animals in the groups.

We also evaluated the amount of water (a general average of 66.7 mL, *Figure 2*) and food (a general average of 29.1 g, *Figure 3*) consumed by the SD rats over the span of the experiment design, which showed not significant difference between any of the groups measured in the study. Results correspond to the historical values observed for rats of this category in our facilities (Sosa *et al.* 2005; Fariñas *et al.* 2014; Oliva *et al.* 2019).
The body temperature recorded during the different evaluations carried out post-inoculation did not show significant differences between groups of treatments (*Figure 4*), it behaved within the physiological ranges reported for the species (CCAC 1984). In order to assess the inflammation induced by the formulations adjuvanted vaccines

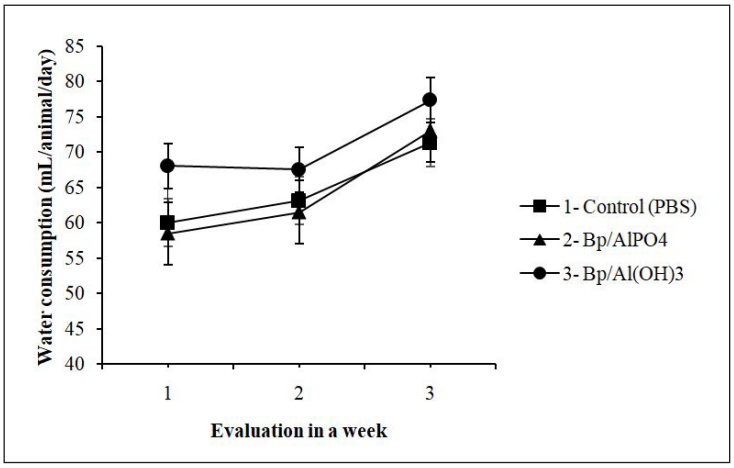

Figure 2. Water consumption time course ofrats with formulations adjuvants *B. pertussis* vaccine. Each value represents the average ± SEM of the 10 animals in the groups.

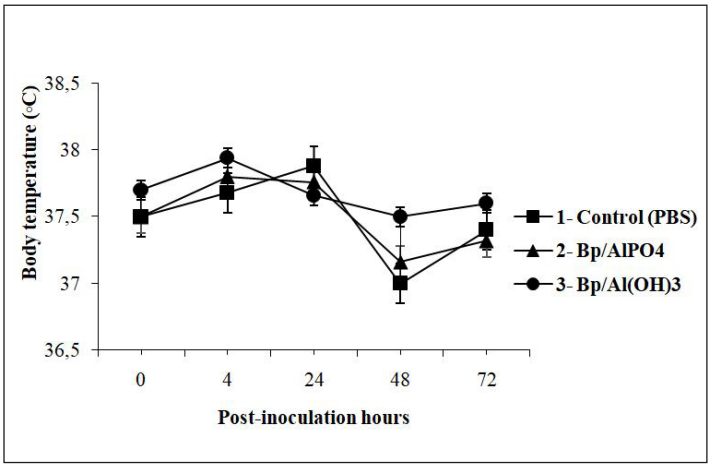

Figure 4. Corporal temperature time course of rats with formulations adjuvants *B. pertussis* vaccine. Each value represents the average ± SEM of the 10 animals in the groups.

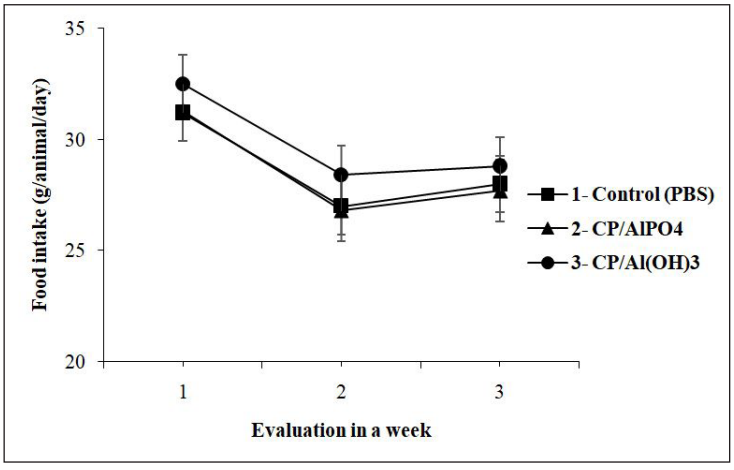

Figure 3. Food consumption time course of rats with formulations adjuvants *B. pertussis* vaccine. Each value represents the average ± SEM of the 10 animals in the groups.

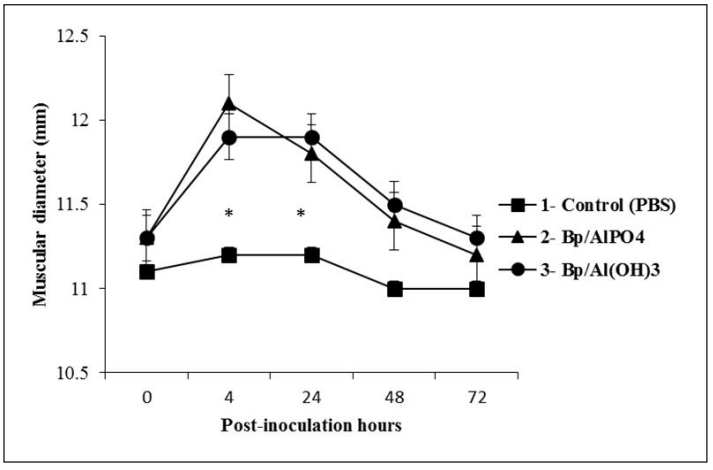

Figure 5. Muscle diameter time course of rats with formulations adjuvants *B. pertussis* vaccine. Each value represents the average ± SEM of the 10 animals in the groups. Asterisk (*) indicates significant difference from the rest of the groups ($p < 0.05$).

Bp, administered intramuscularly as element of reactogenicity and local response, the muscle diameter of the legs was measured before and after receiving the IM vaccination. We found significant differences between controls and treated with formulations of $AlPO_4$ and $AL(OH)_3$ with Bp antigens at 4 and 24 hours post-inoculation and not between the formulations (*Figure 5*).

The dermal irritability test performed by the Draize method allowed discarding through the skin readings at the site of administration of the two formulations; the absence of erythema, edemas, eschar or papules, being the scale of dermal irritation for both 0,0.

In general, macroscopic studies performed on all organs and systems for each of the SD rats studied did not show any lesions suggesting acute toxicity. At the site of inoculation, abscessed white-grayish formations were observed in the animals receiving the formulations. It may be related to the adjuvants $AlPO_4$ and $AL(OH)_3$, as this is a reported characteristic of them (Sosa *et al.* 2005; Oliva *et al.* 2019). Also, the deep popliteal and inguinal lymph nodes were seen size increase.

Discussion

From the past century, aluminum in different form has been used to improve the immunogenicity of vaccines and it's the most studied and used in combined vaccines as adjuvant and current evidence supports the safety of aluminum adjuvants in vaccines (Eickhoff and Myers 2002; CDC 2019; DeStefano *et al.* 2019). Particularly, Bp vaccines have a certain reactogenicity attribution, especially those of whole cell (WHO 2015). However, when Bp are combined with other Ag, be of whole cell or acellular, his reactogenicity could be decrease or be diluted with other Ag present in formulation, unlike when they are the only ones present. For other side, aluminum as component and adjuvant for vaccines continues to be recognized by experts and the World Health Organization of low risk (WHO 2012; CDC 2019) and they are important as strategic part of the news formulations of combined vaccine.

The local response or reactogenicity of vaccines in clinical trial is a critical element, more for combined vaccines, because can have many different Ag in one formulation. Reason why preclinical toxicity essays become important and with highs predictive value to human test. In addition, in these preclinical essays, also taken in account systemic effects, like body weight, fever, and others.

Effects on animal body weight usually manifest in either a decrease in gain compared to controls and placebo or absolute losses of body weight, and are dependent on the toxicity of the product tested. This is a determinant parameter in toxicological studies. The evaluation of body temperature, local inflammation and redness of the skin at the injection site for the IM route can be related to some kind of systemic effect and they are parameters considered predictive for clinical trials (Oliva *et al.* 2019); where observations is based in expected and unexpected adverse events, which include fever, headache, pain at the site of administration, redness, abdominal pain, vomiting, diarrhea, among others (Ochoa *et al.* 2006; García *et al.* 2011).

In our case, both adjuvant formulations ($AlPO_4$ and $Al(OH)_3$) Bp vaccine can be considered of low reactogenicity similar to current combined vaccines with Bp as component Ag. Because, when a fever (>38°C) is induced by some vaccines by intramuscular injection it is registered within the first 24 hours (Hamilton 2013). We measured the temperature of the rats before and after vaccination during 72 hours to determine whether fever was induced. While a traditional fever was never induced in our study, generally in combined vaccines this can be seen and is an element to keep in mind.

The increase in muscle volume that implies difference between controls and vaccinates is explained by the inflammatory process caused by hydroxide and aluminum phosphate used as adjuvants (Sosa *et al.* 2005; Fariñas *et al.* 2014; Oliva *et al.* 2019), and by the Bp itself, which due to its inactivated cell nature generates a local inflammatory response. This reaction lasted approximately 24 hours, since at 48 hours there were no differences between controls and treated. However, the absence of symptoms of lameness, pain or some type of locomotion difficulty evidence for both adjuvants Bp, suggest that vaccine formulations are tolerable and support more the evidences on aluminum safe (DeStefano *et al.* 2019). Moreover, Draize method scale through skin lectured, classify these formulations as potentially non-irritating to the skin, according to the criteria established in the literature (Draize 1944; OECD 2004). Other similar studies made for us, show usefulness of this method (Oliva *et al.* 2019) and comply with the OECD guidelines (OECD 2004).

The fact that male SD rats did not experience any adverse side effects nor where there changes in any of the organs evaluated in gross necropsy, would suggest that the formulations does not toxic. Nevertheless, on the local and inflammatory response of the lymphatic organs (popliteal and inguinal lymph nodes) and immune system were activated, showing an increase similar to other vaccines, being recognized as a normal immunological event with a direct related to the adjuvant and Ag (Sosa *et al.* 2005; Ahrendt *et al.* 2008; Sainte-Marie 2010; Bacardí *et al.* 2013; Oliva *et al.* 2019; Tamargo *et al.* 2019).

Together, the data suggests that not only low reactogenicity of vaccines formulations of inactivated Bp cells adjuvanted in phosphate or aluminum hydroxide were similar, but it is also potentially non-toxic in a male SD rat model. Also, suggest that this antigenic component can be used in combined vaccines with either adjuvant, not implying this any change in their reactogenicity.

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