Brief report

*Bacillus atrophaeus* inactivated spores as a potential adjuvant for veterinary rabies vaccine

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**ABSTRACT**

Rabies is a viral encephalitis, nearly always fatal, but preventable through vaccines. Rabid animal bite is the prime transmission act, while veterinary vaccination is one of the best strategies for rabies general prevention. Aluminum compounds and saponin are the commercial adjuvants used for this vaccine nowadays. Nevertheless, aluminum compounds can provoke undesired side effects and saponin has a narrow activity range without toxicity. *B. atrophaeus* inactivated spores (BAIS), with or without saponin, were then used as an alternative to boost the inactivated rabies virus response. BAIS was as effective as saponin in augmenting antibody titers, but combination of both adjuvants doubled the titers raised by them individually. The combined adjuvant formulation maintained viability for 21 months when stored at 4–8 °C. Overall, BAIS was demonstrated as a viable alternative to commercial adjuvants, while its combination with saponin resulted in even higher vaccine potency with good stability.

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1. **Introduction**

Rabies is a viral encephalitis caused by a single strand RNA enveloped virus. This non-treatable mammal infection has the highest lethality rate of all known infectious diseases \[1,2\]. The most common form of transmission is through bites from rabid animals \[2\]. Fortunately, rabies can be prevented with efficacious inactivated vaccines available for both pre and post exposure \[3\]. Currently, the most effective way to drastically decrease the infection rates is through veterinary vaccination \[2\]. The rabies vaccines for animals are administered by parenteral or oral route, with inactivated or attenuated rabies virus, and may contain aluminum compounds and saponin as adjuvants. Independent of the vaccine type used, animals have to be vaccinated several times during their lifetime (1–3 years intervals) \[1\], an expensive and not always executed practice.

Saponin is currently a licensed adjuvant for veterinary rabies vaccines. Its adjuvant mechanism is not fully understood, but results in strong immunomodulation \[4\]. However, the narrow concentration range that presents immunomodulation without toxicity limits its individual application \[5\].

Experimentally, a large sort of techniques have been applied to improve veterinary rabies vaccine, including new adjuvants \[6,7\]. This work proposes another innovative approach directed to vaccine improvement, combining *Bacillus atrophaeus* inactivated spores (BAIS) and saponin with rabies inactivated virus.

The non-pathogenic strain *B. atrophaeus* ATCC 9372 was originally classified as *Bacillus subtilis*, but the production of a pigment under certain culture conditions, absent in *B. subtilis* subspecies, led to the reclassification. Notwithstanding, except for pigment production, both species are indistinguishable by standard characterization methods \[8\]. Recently, *B. subtilis* inactivated spores (BSIS) were presented as an effective microparticle adjuvant. The approach was effective for induction of higher IgG titers against tetanus toxoid than the titers induced by the toxoid alone \[9\]. Live or inactivated spores were both capable of inducing an effective cellular and humoral immune response against the tested antigens \[10\]. The successful application of BSIS as a vaccine adjuvant stimulated the use of the highly similar strain *B. atrophaeus* as an adjuvant candidate for rabies veterinary vaccine.

2. **Methods**

2.1. **Reference materials and reagents**

The second International Standard for Human Rabies Immunoglobulin determined by the World Health Organization (WHO 2nd HRIG), containing 30 international units/mL (IU/mL), was obtained from the National Institute for Biological Standards and Control (NIBSC, United Kingdom). *In vitro detection*
of Rabies virus by immunofluorescence was performed with fluorescein labeled anti-rabies nucleocapsid conjugate (Bio-rad, USA). Saponin adjuvant extracted from *Quillaja saponaria* (QSA) was donated by Beraca, Brazil.

### 2.2. Inactivated *B. atrophaeus* spores (BAIS)

*B. atrophaeus* ATCC 9372 was grown and sporulated as previously published [11]. Briefly, vegetative cells were sporulated for 3 days/37°C in Plate Count Agar (PCA) medium, supplemented with 0.1% soluble starch, 0.05% magnesium sulfate, 0.05% manganese sulfate and 0.05% calcium chloride. Spores were then harvested and suspended in 0.2 M calcium acetate solution (pH 9.7). After centrifugation (2000 × g/30 min), four successive washes were performed with 0.02 M calcium acetate solution (pH 9.7). The spores were inactivated by autoclaving (121°C, 20 min), centrifuged (2000 × g/30 min), washed and resuspended in phosphate buffer (PBS pH 7.2). The concentration of the suspension was adjusted to 4 × 10^8 spores/mL. Quantification of viable spores was done before and after autoclaving, by plating and incubating spores aliquots serially diluted in TSA (37°C/48 h), followed by counting of colony-forming units. In order to assure inactivation after autoclaving, the incubation period was extended to 7 days, with daily monitoring. The spores were considered inactivated when no colonies were observed, independent of the dilution used.

### 2.3. Rabies virus

The inactivated rabies virus used for vaccination was rabies fixed PV Pasteur virus (Pasteur Institute, France), adapted and extracted from BHK-21 cells (C13 – ATCC CCL-10) as published before [12] and quantified by Antigen Binding Test (ABT) [13]. The stock solution contained 8IU/mL and 1 mg/mL protein content. Challenge virus strain used was CVS-11 (Pasteur Institute, France), which was produced in BHK-21 cells and provided by Instituto Nacional de Controle de Qualidade de Saúde (INQCS, Brazil).

### 2.4. Determination of antigen adsorption by inactivated spores

Log serial dilutions of inactivated spores suspension in PBS pH 7.2 were mixed with rabies inactivated virus (1 mg/mL protein content), incubated (2 h/4°C) and centrifuged (2000 × g/30 min). The supernatant containing the non-adorsed virus was collected and assayed for protein content by Bicinchoninic Acid Assay Kit (Merck, USA).

### 2.5. Determination of rabies cell culture infective dose 50% (CCID50%)

Rabies CVS titration was done to calculate the CCID50. Eight aliquots of stock CVS were serially diluted (log-dilution). BHK21 cells diluted in Eagle’s Minimum Essential Medium (EMEM) and 2.5% fetal bovine serum were added to each well and incubated overnight. Cells were then washed and fixed with cold acetone, dried at room temperature and stained with FITC anti-rabies antibody. Plates were analyzed on an inverted fluorescence microscope (8 fields per well) at 200× magnification. Each analyzed field was classified as positive (virus infection) or negative, based on fluorescence spots. Calculation of CCID50% was done according to Reed and Muench method [14].

### 2.6. Immunization and blood collection

The animal work described here was in accordance with the animal care procedures described in NIH OACU-ARAC guidelines, and approved by the Institutional Animal Care and Use Committee from Butantan Institute (protocol 69/2007). Five-week-old male Swiss mice were obtained from Butantan Institute (SP, Brazil). Six test groups of 8 mice each were inoculated by the intraperitoneal route (27 G × ½ needle) with 0.5 mL of the following preparations: Group 1 with saline phosphate buffer pH 7.2 (negative control group); group 2 with inactivated rabies virus (1 IU/dose); group 3 with BAIS (10^6 spores/dose); group 4 with inactivated rabies virus (1 IU/dose) and BAIS (10^6 spores/dose); group 5 with inactivated rabies virus (1 IU/dose), QSA (70 μg/dose) and group 6 with inactivated rabies virus (1 IU/dose), BAIS (10^6 spores/dose), QSA (70 μg/dose). Two additional groups were inoculated, one with 10^8 spores/dose and another with 10^10 spores/dose, for toxicity evaluation. Mice were inoculated two times (days 0 and 14) and blood collected by submandibular vein (4 mm lancet) on day 21. For stability purposes, 3 batches of the formulation containing inactivated rabies virus, BAIS and QSA were manufactured and stored at 4–8°C. A group of eight mice to each batch in each evaluation time point were immunized and blood collected as described above.

### 2.7. Blood processing and antibody analysis

Blood samples were incubated for 1 h at room temperature and centrifuged for 20 min/1500 × g/4°C. The supernatant (serum) collected was analyzed by ELISA to determine anti-rabies glycoprotein antibody titers using Platelia Rabies II kit (Bio-Rad) [15]. Sample serum was also heat-inactivated (56°C for 30 min) and analyzed through RFFIT, as described before [12], to determine anti-rabies neutralizing antibody titers. RFFIT is listed by the European Pharmacopoeia as one of the methods for potency evaluation of rabies veterinary vaccines [16]. Triplicates of sample serum and WHO 2nd HRIG (standard) were serially diluted in 96 well plates. CVS virus was then distributed to all wells (200 × CCID50), except negative control wells, and the plate incubated for 90 min/37°C/5% CO2. After incubation, BHK21 cells diluted in Eagle’s Minimum Essential Medium (EMEM) and 2.5% fetal bovine serum were added to each well and incubated overnight. Cells were then washed and fixed with cold acetone, dried at room temperature and stained with FITC anti-rabies antibody. Plates were analyzed with an inverted fluorescence microscope (8 fields per well) at 200× magnification. Each analyzed field was classified as positive (virus infection) or negative (virus neutralization) based on fluorescence spots. The serum dilution that reduces the number of fields containing infected cells by 50% (ED50) was calculated using the same method applied to CCID50%. Antibody neutralizing titers were calculated based on ED50 from the standard serum, expressed as International Units per milliliter (IU/mL).

### 2.8. Statistical analysis

Significant statistical differences between group means were analyzed by Student’s t test (α = 0.05, two tailed).

### 3. Results and discussion

#### 3.1. Toxicity evaluation of inoculated mice

Toxicity was evaluated on a daily basis through the following parameters: physiological changes (significant weight gain or loss, diarrhea), behavioral changes (fatigue, hyperactivity) or physical changes (hair loss, excessive local inflammation). The monitoring process was followed until 21 days after the second inoculation. The mice groups inoculated with 1 × 10^6, 1 × 10^7 or 1 × 10^8 inactivated spores/dose presented no apparent signs of toxicity. The highest dose tested was 10,000 times higher than the dose used in the putative rabies vaccine.
Saponin toxicity in Swiss mice was evaluated by our group before [5]. The highest saponin concentration without intoxication signs was found to be 90 μg/dose. As a safety precaution, we used 70 μg/dose in the following experiments.

The same toxicity parameters were monitored for all immunized groups, however no signs of toxicity or abnormal local reactions were observed. Although no symptoms were detectable, toxicity of the chosen vaccine formulation should further be studied in depth to confirm safety for future application.

3.2. BAIS ability to adsorb antigens

The spores of BSIS were reported as particles able to adsorb antigens [10]. In order to verify if BAIS could have the same property, the adsorption ability of BAIS was tested with the stock solution of rabies inactivated virus (Fig. 1). BAIS were mixed with antigen, centrifuged and the supernatant was tested for protein content. The supernatant of centrifuged BAIS alone presented no detectable protein content. The centrifuged antigen alone had 1 mg/mL of protein content. When serial log dilutions of BAIS were mixed with the inactivated virus, a linear correlation between log dilution and protein content from the supernatant were observed (Fig. 1). The inverse correlation exists within a large concentration range (1 × 10³ to 1 × 10⁶ BAIS), in which the decrease of supernatant protein content means an increase of protein adsorption on spores. BAIS at 1 × 10¹⁰ was enough for full antigen adsorption, as no protein content was detected in the supernatant. The number of spores used for immunization experiments (1 × 10⁶ BAIS) was able to absorb 0.5 mg of antigen. Since the protein content used for antigen inoculation was 0.125 mg (1 UI/dose), the quantity of spores should be enough to absorb the amount of antigen in the suspension. This assumption was confirmed by protein analysis, in which no protein was detected.

3.3. Potency evaluation of putative formulations for inactivated rabies vaccine

Putative rabies vaccines were prepared with or without saponin and BAIS as adjuvants, while inactivated rabies virus was utilized as the source of antigens. In order to determine anti-rabies potency increments due to adjuvant action, outbred mice were inoculated with the formulations and their serum assayed for rabies neutralizing antibodies (Fig. 2). The serum was also analyzed by enzyme-linked immunosorbent assay (ELISA) to confirm the results obtained by the traditional in vitro screening process (Fig. 2). The data from both methods were correlated, as expected [15]. Significant higher titers of antibodies were induced when an adjuvant was inoculated, as compared with the inactivated virus alone (p < 0.01). Nonetheless, the antibody titers from mice immunized with antigen and saponin did not differ from the titers obtained from mice inoculated with antigen and BAIS. When saponin and BAIS were combined with inactivated rabies virus in the same inoculation, anti-rabies glycoprotein antibody and neutralization antibody titers quadruplicated at least (p < 0.001). The exact p values for each comparison are listed in Fig. 2 legend. The improvement achieved shows that although inactivated spores were as effective as saponin, adjuvant combination was even more. Saponin alone could have led to higher antibody titers if it was used more concentrated, but this strategy would have resulted in toxicity as an undesired side effect [5]. A preliminary screening experiment performed by our group showed no gain in adjuvant activity by raising the amount of spores per dose (data not shown). The significant higher potency showed when both adjuvants were combined led us to choose this formula for stability evaluation.

3.4. Stability evaluation

The next step consisted in determining stability of the formulation containing both adjuvants to access its suitability. According to WHO, potency is a stability indicating parameter to measure alterations on the efficacy of a vaccine during a timeline [17]. The stability evaluation was based on potency determination by RFFIT from 3 independent batches, analyzed every trimester from days 10 to 820 after batch manufacturing (Fig. 3). The potency decreased
progressively, but the first 50% loss occurred in a longer period than the loss of the remaining potency. The seventh trimester was the last one in which all batches retained potency values above the minimum required for vaccine commercialization (1 IU/mL) [16], hence the shelf life of this formulation can be stated as 21 months. Additional stability parameters were evaluated: visual stability (color change, turbidity or precipitation) and pH monitoring. None of the cited parameters changed over the analyzed period.

4. Conclusion

This work demonstrates that BAIS can absorb inactivated rabies virus and improve murine antibody titers raised by this antigen. In addition to this fact, the spores did not provoke any visual intoxication signs. The commercial saponin admixed with the experimental adjuvant BAIS resulted in excellent vaccine potency, superior to the results obtained with a single adjuvant. The formulation was viable for 21 months at 4–8°C, while most commercial veterinary vaccines have a shelf life of 12 months. Additional studies are needed to confirm the safety of this formulation containing two adjuvants. Further studies with different animal species can confirm the broadness of efficacy for this putative vaccine and future practical application.

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References


Fig. 3. Stability evaluation of the putative rabies vaccine containing inactivated rabies virus, saponin and BAIS. Three batches of the chosen formulation were manufactured and stored at 4–8°C. Serum potency (RFIFIT) of groups of eight immunized Swiss mice was determined for each batch every trimester of storage, from days 10 to 820 after batch manufacturing. Values represented in the graph are the mean of the three batches with error bars for standard deviation.