Veterinary Immunology and Immunopathology 169 (2016) 63–67

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short communication

Experimental evaluation of inactivated and live attenuated vaccines against Mycoplasma mycoides subsp. mycoides

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ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form
25 November 2015
Accepted 11 December 2015

Keywords:
Contagious bovine pleuropneumonia
Inactivated vaccine
Mycoplasma mycoides subsp. mycoides

ABSTRACT

The current control method for contagious bovine pleuropneumonia (CBPP) in Africa is vaccination with a live, attenuated strain of *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*). However, this method is not very efficient and often causes serious adverse reactions. Several studies have attempted to induce protection using inactivated mycoplasma, but with widely contradictory results. Therefore, we compared the protective capacity of the live T1/44 vaccine with two inactivated preparations of *Mmm* strain Afadé, inoculated with an adjuvant. Protection was measured after a challenge with Afadé. The protection levels were 31%, 80.8% and 74.1% for the formalin-inactivated, heat-inactivated and live attenuated preparations, respectively. These findings indicate that low doses of heat-inactivated *Mmm* can offer protection to a level similar to the current live attenuated (T1/44) vaccine formulation.

1. Introduction

Contagious bovine pleuropneumonia (CBPP) is a respiratory disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides (Mmm)* and listed by the World Organization for Animal Health (OIE, 2014) as one of the most economically important livestock diseases in Africa.

Currently, a live attenuated culture of the causative organism strain T1/44 is used as a vaccine of choice. Although it confers some level of immunity, the T1/44 vaccine has certain drawbacks that include low efficacy (Thiaucourt et al., 2000) and a short duration of immunity. Further, the vaccine causes adverse post-vaccinal reactions at the site of inoculation leading to poor acceptance by farmers (Kusiluka and Sudi, 2003; Sori, 2005). Finally, the vaccine has poor stability (short shelf life), hence the requirement for a cold chain during delivery (Rweyemamu et al., 1995) and there is the possibility of reversion to virulence (Mbulu et al., 2004). For this reason, an efficient inactivated vaccine would be a useful addition to the existing prophylactic measures.

Vaccinations using inactivated vaccines have been successful in a number of mycoplasma diseases including contagious agalactia (Buonavoglia et al., 2008) and contagious caprine pleuropneumonia (Rurangirwa et al., 1987). Similar trials for CBPP with a saponin-inactivated vaccine (Nicholas et al., 2003) and with an Immunostimulating Complex (ISCOM) formulation (Hübschle et al., 2003) have not yielded success.

The inactivation method and quantity of mycoplasma administered may play an important role. Inactivation by heat or by sodium hypochlorite can substantially alter the antigens from *Mycoplasma agalactiae* and hence reduce the immunogenicity (Tola et al., 1999). However, two doses of 20 ml at a protein concentration of 14.5 mg/ml of heat inactivated mycoplasma formulated with a suitable adjuvant induced immunity against CBPP (Gray et al., 1986). This suggests that mycoplasma may have to be present in large numbers, either alive or dead, to induce a sufficient protective response and confirms that an inactivated vaccine can confer immunity. Protection by the live T_1 strain of *Mmm* has also been shown to be dose dependent, with a low dose of 10^5 mycoplasma conferring low protection, while there was no significant difference

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http://dx.doi.org/10.1016/j.vetimm.2015.12.006

between doses of 10^7 and 10^9 (Gilbert and Windsor, 1971; Masiga et al., 1978; Thiaucourt et al., 2000).

Owing to the discrepancies in reports on the capacity of inactivated mycoplasma to protect against CBPP, three vaccines formulations were assessed for their protective capability: formalin inactivated (Garba and Terry, 1986) and heat inactivated (Gray et al., 1986) vaccines were compared with the live attenuated T1/44 vaccine. The main purpose of the study was to evaluate the efficacy of inactivated mycoplasma and compare formalin-fixed and heat-inactivated formulations, using equal quantities of antigen. We used the vaccine preparations that yielded the best results in the previous studies.

2. Materials and methods

2.1. Cattle

Forty Boran cattle (*Bos indicus*) between 8 and 10 months of age were obtained from the International Livestock Research Institute (ILRI) ranch in Kapiti, a CBPP-free region in Kenya, and transported to an isolation unit at Kenya Agricultural and Livestock Research Organisation, Veterinary Science Research Institute (KALRO-VSRI) - Muguga. During the whole period of the experiment, animals were handled according to Kenya legislation for animal experimentation and VSRI Animal Welfare Committee regulations (Approval No. KALRO/VRC/IACUC/2/00122010).

2.2. Vaccine preparation

2.2.1. Formalin inactivation

The vaccine was prepared as previously described (Garba and Terry, 1986). Briefly, the vaccine was prepared from a pure 4-day old culture of the Afadé strain using pleuropneumonia-like organism media (Difco™ PPLO Broth) and harvested by centrifugation at 12,000 g and reconstituted to a concentration of 10^{10} colony forming units per millilitre, as assessed by colour changing units (CCU) (Litamoi et al., 1996). The culture was inactivated by adding 0.7% (v/v) of formaldehyde (BDH Chemicals Ltd, Poole, UK) and incubated overnight at 37 °C. The suspension contained 3 mg/ml of protein as determined using the Bicinchoninic Acid method. The preparation was then stored at +4 °C until used. The final product was obtained by emulsifying equal volumes of inactivated culture and of Freund's Incomplete Adjuvant (FIA; Difco).

2.2.2. Heat inactivation

Preparation of the vaccine was done as described (Gray et al., 1986). The Afadé strain of *Mmm* was grown for 4 days in PPLO media (Difco) to a concentration of 10^{10} CCU/ml and centrifuged at 12,000 g. The pellet was washed three times in phosphate buffered saline (PBS). and re-suspended in 10 ml of the same solution. Protein concentration was then determined using the Bicinchoninic Acid Assay. This suspension was adjusted to contain 3 mg/ml of mycoplasma protein. Mycoplasma were killed by heating in a water bath at 56 °C for 30 min. To determine if there was any viable mycoplasma left, 1 ml of the suspension was dispensed in 9 ml growth media and observed for any colour change. No colour change was observed after 8 days and hence the killed mycoplasma was then kept at −20 °C until the day of immunization. On the day of vaccination, an equal volume of Freund's Complete Adjuvant (FCA; Difco) was added and mixed by means of an emulsion mixer. The drops of mixed emulsion did not disperse on the surface of PBS.

2.2.3. Live attenuated (T1/44)

Contavax (B/No. 01/2012) (Kenya Veterinary Vaccine Production Institute) at a concentration of 10^7 live Mycoplasma per animal was used. Immunization was done as instructed by the manufacturer.

2.3. Experimental design

Cattle were randomly divided into four groups of ten animals, one of which comprised the non-immunized control group. On day 0 of the immunizations, groups 1 and 2 were inoculated with heat-inactivated, formalin-inactivated and live attenuated (T1/44) vaccine, respectively. Each animal was subcutaneously vaccinated with 2 ml of the vaccine formulation on the neck. Animals received a primary immunization with Freund's complete adjuvant for the heat-inactivated mycoplasma or Freund's incomplete adjuvant for the formalin-fixed mycoplasma. After three weeks, two booster injections, separated by 2 months, were delivered with the inactivated mycoplasma mixed with Freund's incomplete adjuvant in both groups. Group 3 was inoculated with a single dose of live T1/44 on day 0 of the immunizations. During the experiment five animals died of causes not related to CBPP.

2.4. Challenge and clinical observations

Three weeks post the second booster administration, cattle were challenged by endotracheal intubation of 60 ml of *Mmm* culture (approximately 10^9 CCU/ml) following the method described (Nkando et al., 2010).

The cattle were restrained in a crush daily at the same hour (09:00–10:00 am) for clinical observation. Rectal temperatures, coughing and general condition were recorded daily.

2.5. Serological examination

The animals were bled immediately before vaccination and at weekly intervals during the trial period. Blood samples were taken from the jugular vein into vacutainer tubes and allowed to clot at room temperature overnight. Following separation by centrifugation, serum samples were collected and stored at −20 °C until examined by the complement fixation test (CFT) (Campbell and Turner, 1953).

2.6. Post-mortem examination, mycoplasma isolation and lesion scoring

Post-mortem was carried out on all the animals 56 days after challenge. The cattle were euthanized by captive bolt and then exsanguinated. Lungs were examined for CBPP lesions including encapsulation, consolidation, fibrous adhesion and sequestration. The type and size of the lesion was recorded.

Lung tissues were collected between the lesion and the grossly normal tissue and stored at -20 °C until culturing for mycoplasma was done. Culturing of *Mmm* from the lungs was done by incubating a small piece of the lung tissue in Gourlay broth media (with penicillin and thallium acetate) at 37 °C in a humidified 5% CO_2 incubator. One milliliter of the suspension was titrated in a dilution series (1:10, 1:20, 1:30 and 1:40) after one day of growth. From these dilutions, 0.2 ml was then dropped onto agar plate with Gourlay media and incubated at 37 °C. Morphological features and typical "fried egg" appearance checked at days 1, 5 and 10 for *Mmm* colonies.

Lesion scoring (Hudson and Turner, 1963) was used to determine the severity of the disease in each animal. This scoring was done as follows;