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

Martin Mwirigi[a,b,*], Isabel Nkando[c], Racheal Aye[a], Reuben Soi[b], Horace Ochanda[d], Emil Berberov[e], Andrew Potter[e], Volker Gerdts[e], Jose Perez-Casal[e], Jan Naessens[a], Hezron Wesonga[c]

[a] International Livestock Research Institute, P.O. Box 30709-00100, Nairobi, Kenya
[b] Kenya Agricultural and Livestock Research Organisation, Biotechnology Research Institute, P.O. Box 14733 00800, Nairobi, Kenya
[c] Kenya Agricultural and Livestock Research Organisation, Veterinary Science Research Institute, P.O. Box 32-00902, Kikuyu, Kenya
[d] University of Nairobi, P.O. Box 30197, Nairobi, Kenya
[e] Vaccine and Infectious Disease Organization – International Vaccine Centre, 120 Veterinary Road Saskatoon, SK S7N 5E3, Saskatoon, Canada

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

* Corresponding author. Tel.: +254 722 325 524.
*E-mail addresses:* martin.mwirigi@kalro.org, kiogoramk@yahoo.com (M. Mwirigi).

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;

(1) Presence of only encapsulated, resolving or fibrous lesion or pleural adhesion: 1 - Or if presence of other type of lesion like consolidation, necrosis or sequestration: 2
(2) *Mmm* isolated: 2

The resulting sum of the score (maximum 4) was multiplied by a factor depending on the lesion size (1: < 5 cm; 2: > 5 and < 20 cm; 3: > 20 cm in diameter). Protection rate was calculated as follows:

Vaccine efficacy = 1 − [mean score of vaccinates/mean score of controls] × 100

### 2.7. Statistical analysis

Analysis of variance (ANOVA) was used to determine if there were differences in CFT titres following vaccination between the three vaccinated groups. Pearson correlation coefficient was used to analyse relationship between CFT titres and pathology score. Pathology scores were evaluated to determine if there was a difference in protection afforded by the three vaccines. Differences between mean pathology scores in the four groups (vaccinates and controls) were also analysed using ANOVA. To evaluate differences between the antibody response as revealed by CFT was performed using Chi-square ($\chi^2$) test. Prism statistical software (6th edition) was used in the analysis.

### 2.8. Results and Discussion

This study compared the protective capacity of the live T1/44 vaccine with two inactivated preparations of *Mmm* strain Afadé, formulated with adjuvants. Both the heat (Gray et al., 1986b) and formalin (Garba and Terry, 1986) inactivated vaccine formulations had been shown to have complete protective capacity. The heat-inactivated mycoplasma emulsified with complete Freund's adjuvant, was used at a concentration of 14.5 mg of protein/ml and 10 ml were used during vaccination (Gray et al., 1986b). The formalin inactivated 0.5 ml dose of $10^{10}$ mycoplasma/ml was protective against CBPP. In the present experiment a uniform dose of 3 mg/ml of proteins was used in the heat and formalin inactivated vaccine formulations while T1/44 was used as instructed by the manufacturer.

### 2.9. Clinical observations

Animals under study had no clinical signs prior to challenge. Clinical responses observed after challenge included cough, nasal discharge and fever (in this study considered to be ≥39.5 °C). A high number of animals in all the groups experienced respiratory distress characterised by coughing (Table 1). This was observed from the second week post intubation except for the group receiving formalin-fixed organisms that experienced symptoms from the

**Table 1**
Clinical and pathological results in animals vaccinated with inactivated and T1/44 vaccines.

| Vaccination group | Animal No. | Respiratory distress (coughing) observed | *Mmm* isolated | Lung/Thoracic cavity lesions (cm) | Pathology Score |
|---|---|---|---|---|---|
| Heat-inactivated | 819 | Yes | −ve | No lesion | 0 |
| | 802 | Yes | −ve | No lesion | 0 |
| | 825 | No | −ve | No lesion | 0 |
| | 815 | Yes | −ve | Cons | 2 |
| | 813 | No | −ve | No lesion | 0 |
| | 803 | Yes | −ve | Fib adhe | 1 |
| | 824 | Yes | −ve | No lesion | 0 |
| | 804 | Yes | −ve | Fib tags | 1 |
| | 806 | Yes | −ve | Fib adhe<br>No lesion | 1 |
| Formalin-inactivated | 807 | Yes | +ve | Seq (3x3) | 4 |
| | 827 | Yes | −ve | No lesion | 0 |
| | 814 | Yes | −ve | Cons | 2 |
| | 800 | Yes | −ve | No lesion | 0 |
| | 801 | Yes | −ve | Cons | 2 |
| | 823 | Yes | +ve | seq (10x7) | 6 |
| | 810 | No | −ve | No lesion | 0 |
| Live attenuated (T1/44) | 808 | Yes | +ve | No lesion | 2 |
| | 812 | No | −ve | Cons | 2 |
| | 811 | Yes | −ve | No lesion | 0 |
| | 805 | No | −ve | No lesion | 0 |
| | 816 | Yes | +ve | No lesion | 2 |
| | 828 | Yes | −ve | No lesion | 0 |
| | 820 | Yes | −ve | No lesion | 0 |
| | 829 | No | +ve | No lesion | 2 |
| Control group | 842 | No | +ve | cons | 4 |
| | 843 | No | +ve | No lesion | 2 |
| | 844 | No | +ve | Fib adhe, hep | 4 |
| | 990 | Yes | +ve | Fib adhe, cons | 4 |
| | 991 | Yes | +ve | cons | 4 |
| | 992 | Yes | +ve | cons | 4 |
| | 993 | Yes | +ve | No lesion | 2 |
| | 994 | Yes | −ve | Res les | 1 |
| | 995 | Yes | −ve | No lesion | 0 |
| | 996 | No | −ve | cons | 2 |

Cons = consolidation; fib adhe = fibrous adhesion; res adhe = resolving adhesion, Res les = Resolving lesion; cong = congestion; Seq = sequestration.

**Table 2**
Serology after vaccination and challenge.

| Vaccination group | Animal No. | Highest CF titre after vaccination and before challenge | Highest CF titre after challenge |
|---|---|---|---|
| Heat-inactivated | 819 | 1:20 | 1:80 |
| | 802 | 1:40 | 1:20 |
| | 825 | 1:40 | 1:40 |
| | 815 | 1:40 | 1:40 |
| | 813 | 1:40 | 1:20 |
| | 803 | 1:40 | 1:20 |
| | 824 | 1:40 | 1:20 |
| | 804 | 1:40 | 1:20 |
| | 806 | 1:20 | 1:20 |
| Formalin-inactivated | 807 | 1:40 | 1:40 |
| | 827 | 1:40 | 1:40 |
| | 814 | 1:10 | 1:40 |
| | 800 | 0 | 0 |
| | 801 | 1:80 | 1:40 |
| | 823 | 1:20 | 1:40 |
| | 810 | 0 | 0 |
| Live attenuated (T1/44) | 808 | 1:10 | 0 |
| | 812 | 0 | 0 |
| | 811 | 0 | 0 |
| | 805 | 1:20 | 1:40 |
| | 816 | 0 | 1:20 |
| | 828 | 0 | 1:40 |
| | 820 | 0 | 0 |
| | 829 | 1:20 | 0 |
| Control group | 842 | – | 0 |
| | 843 | – | 0 |
| | 844 | – | 0 |
| | 990 | – | 0 |
| | 991 | – | 1:80 |
| | 992 | – | 0 |
| | 993 | – | 0 |
| | 994 | – | 0 |
| | 995 | – | 1:160 |
| | 996 | – | 0 |

fourth week onward. Major differences in clinical signs among groups were not observed.

One animal in the control group developed fever for five days. The rest of the animals recorded rectal temperatures that were within normal range, suggesting that infection may not have been severe.

### 2.10. Antibody responses post vaccination and post challenge

Antibody (Ab) responses following vaccination and after challenge were measured using CFT. The highest Ab titres recorded during vaccination and after challenge are shown in Table 2. Following vaccination seroconversion was observed in 9/9 (100%), 5/7(71%) and 3/8 (37.5%) animals immunized with heat-inactivated, formalin-inactivated and live attenuated (T1/44) vaccines.

Seroconversion was first detected one week and three weeks post vaccination for the animals that received inactivated and live attenuated vaccine, respectively. The CFT titres were significantly higher after vaccination with the inactivated formulations as compared with the T1/44 vaccine. No significant antibody titre differences were observed between post-vaccination and post-challenge sera. No correlation between antibody titres before challenge and pathological scores at post-mortem was observed.

### 2.11. Pathology and vaccine efficacy

The clinical and pathological observations after challenge in animals vaccinated with inactivated and T1/44 vaccines are shown in Table 1. Post-mortem examination revealed pathological lesions typical of CBPP including consolidation and hepatization of the lung parenchyma and pleuritis, and well-developed sequestra. Fibrous adhesions of the parietal and visceral pleurae and kidney congestion were also observed. Mycoplasma were isolated from animals in the non-vaccinated (7/10), formalin-inactivated (2/7) and T1/44 (3/8) but not in the heat-inactivated group.

The highest number of lung lesions were observed in the control group (8/10), followed by the formalin-inactivated group (4/7), heat-inactivated (3/9) and live attenuated groups (1/10), respectively. Average scores for lesion size were higher in non-vaccinated animals (Table 3). Sequestra formation was recorded only in two animals that had been vaccinated with the formalin-inactivated vaccine.

Vaccine efficacy for the three vaccines, calculated using average Hudson scores, is shown in Table 3. All the vaccines tested offered partial protection against CBPP. The protection rate for the formalin-inactivated, heat-inactivated and live attenuated groups were 31%, 80.8% and 74.1%, respectively. Higher incidences of bronchiopneumonia were observed in the non-vaccinated group than in the vaccinated groups, an indication that severity of the disease was less in vaccinated animals. In the non-vaccinated group, more animals had severe pathology and incidence of mycoplasma isolation from tissues. Significant protection was offered by heat inactivated (80.8%) and live attenuated (74.1%) mycoplasma and found to be comparable to the protection rates of 50%–80% observed previously (Gilbert F. R and Windsor R. 1971; Thiaucourt et al., 2000: Nkando et al., 2012). Although formalin-inactivated Mmm had shown protection previously (Garba & Terry, 1986), protection achieved in our experiment was very low (31%). We used the Afadé strain for immunization instead of Gladysdale, but this is unlikely to be the reason for the different outcome with the previous studies (Garba and Terry, 1986), since Mmm strains show little heterogeneity (Fischer et al., 2013). We used a lower amount of antigen, but unfortunately, we cannot compare the antibody titers between the two studies, that might be the reason for the difference in protection rates.

Use of adjuvant in producing a protective immune response to CBPP by killed mycoplasma is critical. In one experiment (Garba et al., 1989), animals immunised with incomplete Freund's adjuvant were significantly better protected after challenge compared to animals immunized with other adjuvants. The protective capacity of the vaccine therefore depends on the type of adjuvant used, as also demonstrated for other mycoplasma of the mycoides group

**Table 3**
Vaccine efficacy of the vaccine formulations.

| Vaccination group | Vaccine formulation | | | |
|---|---|---|---|---|
| | Heat-inactivated | Formalin-fixed | Live attenuated (T1/44) | Control |
| Number of cattle | 9 | 7 | 8 | 10 |
| Average lesion score | 0.56 | 2 | 0.25 | 1.3 |
| Average Hudson score | 0.56 | 2 | 0.75 | 2.9 |
| Vaccine efficacy (%) | 80.8 | 31 | 74.1 | |

(Mulira et al., 1988). Although Freund's adjuvant enhances protection, it is not recommended for animals intended for human consumption since they cause spoilage of meat, chronic inflammation and sterile abscess.

The cattle immunized with formalin-fixed mycoplasma had higher serum titers of specific antibodies than animals immunized with the heat-inactivated mycoplasma, yet were less protected. This suggests that the formulation plays an important role. We do not know at this stage the nature of the protective antigens, and it seems likely that what we measure in CFT does not correspond to protection. No correlation was evident between Ab titers and the severity of CBPP clinical signs or the types and intensity of lung lesions observed at necropsy and confirms findings by others (Mamadou et al., 2006; Nkando et al., 2012; Sacchini et al., 2012).

In conclusion, this study demonstrates that three injections of a vaccine dose of 3 mg protein of heat-inactivated *Mmm* mixed with Freund's complete adjuvant can offer significant protection from *Mmm* infection. The capacity of an inactivated vaccine to protect against disease indicates that a subunit vaccine or an inactivated vaccine against CBPP in a commercially acceptable adjuvant may be possible. However, careful selection of adjuvant should be done taking into consideration the potency, safety and ease of application of the vaccine.

## Acknowledgements

This project was funded by the Canadian International Food Security Research Fund (CIFSRF) grant 106929 of the International Development Research Center (IDRC). Part of the project was also supported by a CSIRO-AUSaid grant (CSI002CBP). The authors thank Eric Gitonga, Ernest Kamau, Eunice Ogugo, Desterio Ouma and Charles Kagwai from KALRO, and Francis Chuma and Joseph Gesharisha from ILRI for technical assistance.

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