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Research paper

Recombinant *Mycoplasma mycoides* proteins elicit protective immune responses against contagious bovine pleuropneumonia☆

Isabel Nkando[a,1], Jose Perez-Casal[b,*,1], Martin Mwirigi[a], Tracy Prysliak[b], Hugh Townsend[b], Emil Berberov[b], Joseph Kuria[d], John Mugambi[a], Reuben Soi[a], Anne Liljander[c], Joerg Jores[c,e], Volker Gerdts[b], Andrew Potter[b], Jan Naessens[c,2], Hezron Wesonga[a,2]

[a] *Kenya Agricultural and Livestock Research Organisation (KALRO), Kaptagat Rd., Loresho, P.O. Box 57811-00200, Nairobi, Kenya*
[b] *Vaccine Infectious Disease Organization—International Vaccine Centre (VIDO-InterVac) 120 Veterinary Rd., Saskatoon, SK S7N 5E3, Canada*
[c] *International Livestock Research Institute (ILRI), P.O. Box 30709, Nairobi 00100 Kenya*
[d] *University of Nairobi, Faculty of Veterinary Medicine, Kapenguria Road off Waiyaki Way, P.O. Box 29053-00625, Nairobi, Kenya*
[e] *Institute of Veterinary Bacteriology, University of Bern, CH-3001 Bern, Switzerland*

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ABSTRACT

Mycoplasma mycoides subsp. *mycoides* (*Mmm*) is the causative agent of contagious bovine pleuropneumonia (CBPP), a devastating respiratory disease mainly affecting cattle in sub-Saharan Africa. The current vaccines are based on live-attenuated *Mmm* strains and present problems with temperature stability, duration of immunity and adverse reactions, thus new vaccines are needed to overcome these issues. We used a reverse vaccinology approach to identify 66 *Mmm* potential vaccine candidates. The selection and grouping of the antigens was based on the presence of specific antibodies in sera from CBPP-positive animals. The antigens were used to immunize male Boran cattle (*Bos indicus*) followed by a challenge with the *Mmm* strain Afadé. Two of the groups immunized with five proteins each showed protection after the *Mmm* challenge (Groups A and C; $P < 0.05$) and in one group (Group C) *Mmm* could not be cultured from lung specimens. A third group (Group N) showed a reduced number of animals with lesions and the cultures for *Mmm* were also negative. While immunization with some of the antigens conferred protection, others may have increased immune-related pathology. This is the first report that *Mmm* recombinant proteins have been successfully used to formulate a prototype vaccine and these results pave the way for the development of a novel commercial vaccine.

1. Introduction

Contagious bovine pleuropneumonia (CBPP) is a highly contagious lung disease in cattle, caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*, Fischer et al., 2012). Currently, the disease is a major constraint to cattle production in Africa causing severe socio-economic consequences (FAO, 2003; Onono et al., 2014; Windsor, 2000). Many countries have successfully controlled the disease by employing a combination of measures, including animal movement restrictions, quarantine, vaccination and elimination of affected herds. The current live vaccine, based on the African *Mmm* strain T1, confers a limited duration of immunity and a rather low efficacy, therefore a better vaccine is a prerequisite for progressive control of CBPP (Jores et al., 2013). Historically CBPP eradication was achieved in Europe and North America through movement control and test and slaughter of suspected cases. In Africa, Botswana, managed to eradicate the disease at a significant cost (Masupu et al., 1997) and Namibia had a successful control campaign based on monitoring cattle movement and vaccination with T1/44 (Huebschle et al., 2003). Many African countries cannot afford to pay financial compensation to the livestock owners. The combination of high cost and the logistical difficulties of movement control between and within pastoral communities, and between small-scale farmers all make the above-mentioned control measures extremely difficult to implement (Kusiluka and Sudi, 2003; Windsor, 2000; Windsor and Wood, 1998).

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* Corresponding author. Fax: +1 3069667478.
E-mail address: jose.perez-casal@usask.ca (J. Perez-Casal).

[1] These two authors contributed equally to this work.
[2] These two authors share last authorship.

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A vaccination campaign would be a viable alternative for CBPP control in Africa (March, 2004; Windsor, 2000). The existing vaccines are live attenuated strains of *Mmm*, known as $T_1$44 and T_1SR both of which confer protection, generally lasting less than one year (Egwu et al., 1996). In addition, the requirements for high dose (Karst, 1971; Masiga and Domenech, 1995; Nicholas et al., 2000; Thiaucourt et al., 2000; Tulasne et al., 1996), cold chain, and the reported adverse effects of $T_1$44 at the site of injection (Daleel, 1971; Provost et al., 1987; Revell, 1973) have resulted in multiple attempts to develop improved vaccines.

Saponin inactivation, which resulted in a successful *Mycoplasma bovis* vaccine (Nicholas et al., 2002), failed in achieving protective immunity after *Mmm*-based immunizations (Nicholas et al., 2003). High doses of heat-inactivated or formalin-fixed *Mmm* did show reduction of pathology (Garba and Terry, 1986; Gray et al., 1986), suggesting that a live pathogen is not essential to induce immunity. Immunization with *Mmm* immune stimulating complexes (ISCOMs) induced both antibody and cell-mediated responses and even caused reduction of mortality in experimentally challenged animals, but failed to affect the formation of lesions (Hubschle et al., 2003).

Immunization with five *Mmm* variable surface proteins postulated to play a role in adherence did not provide protection against CBPP (Hamsten et al., 2010). Immunization with the trans-membrane virulence-related L-α-glycerol-3-phosphate oxidase (GlpO) protein also failed to protect, despite inducing high antibody titers (Mulongo et al., 2013a). High antibody titers to lipoprotein Q (LppQ) have been detected in infected animals (Abdo et al., 2000) and in one study with sera from cattle vaccinated with the live vaccine, higher anti-LppQ titers were found to be correlated with less severe disease (Hamsten et al., 2010). Unfortunately, immunization with LppQ significantly enhanced post-challenge pathology (Mulongo et al., 2015; Nicholas et al., 2003). Enhanced pathology after immunization with a recombinant *M. bovis* protein has also been suggested (Prysliak et al., 2013).

Despite the limited success, the advantages of a subunit vaccine namely potential elimination of cold chain, enhanced efficiency of vaccination and ability to combine antigens with other vaccines are attractive properties. In this study we used antigens previously identified by a reverse vaccinology approach (Perez-Casal et al., 2015) and tested their capacity to induce protection against an experimental CBPP infection. The mycoplasma proteins were prioritized by their likelihood to protect against disease using bioinformatical tools and reactivity with sera from previously infected cattle. The protein list comprised 38 *Mmm* proteins and 28 proteins encoded by *Mycoplasma mycoides* subsp. *capri* (*Mmc*), the causative agent of pneumonia, mastitis and arthritis in goats. The 66 highest ranked proteins were then tested for their capacity to induce an antibody response and T-cell proliferation. In the present study, these 66 recombinant proteins were used to immunize cattle, which were subsequently challenged with *Mmm* to reveal possible protective antigens. This paper reports on the protective effects and immune responses observed with a subset of the antigens used for immunization.

2. Materials and methods

2.1. Ethical considerations

All protocols of this study were designed and performed in strict accordance with the Kenyan legislation for animal experimentation and with the approval of Kenya Agricultural and Livestock Research Organization (KALRO) animal care and use committee (Approval number KALRO/VRC/IACUC/2/00122010)

2.2. Recombinant mycoplasma proteins

The proteins used in the trials were 38 *Mmm* proteins (Protein IDs MSC_xxxx and MMS_Axxxx) and 28 proteins encoded by *Mycoplasma mycoides* subsp. *capri* (*Mmc*, protein IDs YP_004xxxxxx.1; Table 1). All the proteins used in this study were expressed and purified at the Vaccine and Infectious Disease Organization–International Vaccine Centre (VIDO-InterVac), Canada, as previously described (Perez-Casal et al., 2015) and shipped on dry ice to KALRO, Kenya. The prototype vaccines were formulated using pools of five proteins plus CpG-ODN 2007 and 30% Emulsigen™. The placebo groups only received CpG-ODN 2007 and 30% Emulsigen™ (Table 1). The pools of antigens (50 μg of each antigen per inoculation) were the same as described in (Perez-Casal et al., 2015). The proteins were also used for individual testing against serum and cell samples obtained from experimental animals.

2.3. Experimental animals

One hundred and seventy male Boran cattle (*Bos indicus*) aged 2–3 years were purchased from ILRI ranch in Kapiti (a CBPP-free area in Kenya). Before purchase, they were bled, and screened for absence of anti-*Mmm* antibodies using CFT (Etheridge and Buttery, 1976). The CFT-negative animals were transported to the KALRO experimental station and allowed to acclimatize for two months, during which they were drenched with Nilzan plus® (Cooper, Nairobi, Kenya) against helminths, vaccinated against Foot-and-Mouth disease, lumpy skin disease, black quarter, and anthrax; and sprayed with acaricides to control ticks. During the day the cattle grazed in the paddocks while at night they were confined in a brick house. They were fed on hay, mineral supplements and water *ad libitum*.

2.4. Vaccinations and monitoring

Due to logistical challenges this experiment was conducted as three separate trials with 17 groups of cattle (14 immunized with pools of recombinant proteins and 3 immunized with placebo) as follows: cattle were randomly assigned into three sets comprising 60 cattle each for trial 1 and 2, and 50 cattle for trial 3. In each trial, animals were grouped randomly. These groups were designated as group A–Q and received the prototype vaccine formulations as indicated in Table 1. The group designation was consistent between this work and the previous study (Perez-Casal et al., 2015), which tested the immunogenicity of the recombinant antigens (i.e. group "A" in both studies was treated with the same pool of recombinant antigens).

The animals were injected subcutaneously on the right neck with 2 ml of prototype vaccine formulations. Following immunization, they were grazed and housed together, after which a booster immunization was administered on the left neck 21 days later. Rectal temperatures and other clinical parameters were recorded daily. Blood was collected at three-time points (pre-vaccination, post-vaccination and post-challenge) and peripheral blood mononuclear cell (PBMC) were isolated for use in proliferation assays. Samples collected on the day of primary immunization represented day 0 of the trial.

2.5. Mmm *strain and growth conditions*

The *Mmm* strain Afadé (Fischer et al., 2015) was grown in Gourlay's medium (Gourlay, 1964) containing 20% heat-inactivated pig serum, 0.25 mg/ml penicillin, and 0.025% thallium acetate. The medium was stored at 4 °C and used within 14 days. For growing *Mmm*, a 1 ml aliquot of the master seed culture was thawed