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Short communication

First evaluation of an influenza viral vector based *Brucella abortus* vaccine in sheep and goats: Assessment of safety, immunogenicity and protective efficacy against *Brucella melitensis* infection

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ABSTRACT

Previously we developed and evaluated a candidate influenza viral vector based *Brucella abortus* vaccine (Flu-BA) administered with a potent adjuvant Montanide Gel01 in cattle, which was found safe and highly effective. This study was aimed to establish a proof-of-concept of the efficacy of Flu-BA vaccine formulation in sheep and goats. We vaccinated sheep and goats with Flu-BA vaccine and as a positive control vaccinated a group of animals with a commercial *B. melitensis* Rev.1 vaccine. Clinically, both Flu-BA and Rev.1 vaccines were found safe. Serological analysis showed the animals received Flu-BA vaccine did not induce antibody response against *Brucella* Omp16 and L7/L12 proteins during the period of our study (56 days post-initial vaccination, PIV). But observed significant antigen-specific T cell response indicated by increased lymphocyte stimulation index and enhanced secretion of IFN-γ at day 56 PIV in Flu-BA group. The Flu-BA vaccinated animals completely protected 57.1% of sheep and 42.9% of goats against *B. melitensis* 16 M challenge. The severity of brucellosis in terms of infection index and colonization of *Brucella* in tissues was significantly lower in the Flu-BA group compared to negative control animals group. Nevertheless, positive control commercial Rev.1 vaccine provided strong antigen-specific T cell immunity and protection against *B. melitensis* 16 M infection. We conclude that the Flu-BA vaccine induces a significant antigen-specific T-cell response and provides complete protection in approximately 50% of sheep and goats against *B. melitensis* 16 M infection. Further investigations are needed to improve the efficacy of Flu-BA and explore its practical application in small ruminants.
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1. Introduction

Brucellosis is a chronic and infectious disease of ruminants and humans, caused by *Brucella* genus (Bercovich, 2000) which includes at least ten species: *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, *B. neotomae*, *B. cetacea*, *B. pinnipedia*, *B. microti* and *B. inopinata* (Haag et al., 2010). Among them *B. melitensis* is highly pathogenic to humans (Corbel, 1997). We do not have a vaccine for humans and vaccination of animals against brucellosis is one of the most cost-effective measures for protecting the health of humans in endemic areas (Zinsstag et al., 2007), and is the only viable tool in eradication of brucellosis from herds (Garin-Bastuji et al., 1998). Currently, brucellosis in sheep and goats is controlled using *B. melitensis* Rev. 1 live attenuated vaccine. But though it is effective it has many serious drawbacks, such as in a fraction of animals it causes abortion, disease in humans, and stimulates enhanced anti-LPS antibody response which interferes in distinguishing vaccinated from natural infected animals. Furthermore, the strain *B. melitensis* Rev. 1 is resistant to the antibiotic streptomycin used commonly to treat the disease (Schurig et al., 2002). Therefore, development of a safe and effective vaccine against *B. melitensis* to solve this important global problem is warranted.

Earlier as a prophylactic measure against bovine brucellosis (*B. abortus*) we developed a novel Flu-BA vaccine candidate using the

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recombinant influenza viruses of H5N1 and H1N1 subtype to express *Brucella* L7/L12 or Omp16 proteins from the *NS1* (non-structural) gene open reading frame (ORF) (Tabynov, 2016). Our studies demonstrated safety and protective efficacy of Flu-BA vaccine in cattle comparable to commercial vaccines *B. abortus* S19 and RB51 (Tabynov et al., 2014a, 2016a, 2014b; Tabynov et al., 2014c), including in pregnant heifers (Tabynov et al., 2016b). Importantly, the Flu-BA vaccine enables differentiation of vaccinated from infected cattle (Tabynov et al., 2016a), and induces cross-protection against *B. melitensis* infection in pregnant heifers (Tabynov et al., 2015). Therefore, the purpose of this study was to explore efficacy and safety of the Flu-BA vaccine in small ruminants against *B. melitensis* infection.

2. Materials and methods

2.1. Generation of influenza viral vectors

The IVV were generated by a standard reverse genetics method using eight bidirectional plasmids pHW2000. The detailed procedure of generation of IVV were reported previously (Tabynov et al., 2014d). A total of four IVV expressing the *Brucella* L7/L12 or Omp16 proteins were generated: H5N1 (Flu-NS1-124-L7/L12-H5N1, Flu-NS1-124-Omp16-H5N1) and H1N1 (Flu-NS1-124-L7/L12-H1N1 and Flu-NS1-124-Omp16-H1N1).

2.2. Vaccine preparation

Flu-BA vaccine was prepared from the IVV Flu-NS1-124-L7/L12-H5N1, Flu-NS1-124-Omp16-H5N1, Flu-NS1-124-L7/L12-H1N1 and Flu-NS1-124-Omp16-H1N1, grown in 10-day-old chick embryo (CE; Lohmann Tierzucht GmbH, Cuxhaven, Germany) at 34 °C for 48 h. The titer of the IVV was determined using CE as previously described (Tabynov et al., 2012). The titer of the harvested allantoic fluid containing IVV (H5N1 or H1N1) was 6.4–6.5 $\log_{10}$ EID_{50}/ml, and it was combined in a single pool in a 1:1 ratio to obtain the bivalent vaccine formulation. The detailed procedure used to prepare the Flu-BA vaccine was previously described (Tabynov et al., 2016b). Immediately before administration the lyophilized vaccine was resuspended in 10% solution of Montanide Gel01 adjuvant (Seppic, Puteaux, France) in PBS (2 ml per ampoule).

2.3. Vaccination and study design

A total of 21 Degeresskaya crossbred meat and wool breed sheep and 21 Gorno-Altaisk breed of goats aged 3–4 months were procured from tested brucellosis-free flocks to use in this study. All animals were again confirmed seronegative for brucellosis in the laboratory by serology. Sheep and goats were divided into three groups (n = 7 per group): Group I vaccinated with Flu-BA; Group II positive control, vaccinated with a commercial *B. melitensis* Rev.1 vaccine; and Group III negative control, injected with Montanide Gel01 adjuvant in PBS. Animals were vaccinated twice with the Flu-BA vaccine containing IVV subtypes H5N1 (prime vaccination; 6.0 $\log_{10}$ EID_{50}/animal) and H1N1 (booster vaccination; 6.0 $\log_{10}$ EID_{50}/animal) via subcutaneous route at an interval of 28 days. This immunization strategy effectively overcomes the immune background elicited against the viral vector during prime vaccination. Animals in the positive control group were immunized once subcutaneously in the right axillary region with commercial vaccine *B. melitensis* Rev.1 (Antigen, Almaty, Kazakhstan; at dose 2.0×10^6 CFU in 2.0 ml/animal) as per the manufacturer's instructions. Sheep and goats in the negative control group were subcutaneously administered with 2.0 ml of 10% Montanide Gel01 adjuvant in PBS.

This study was carried out in compliance with the national and international laws and guidelines on animal handling. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Research Institute for Biological Safety Problems of the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (Permit Number: 0815/77).

2.4. Assessment of vaccine safety

The safety of the Flu-BA vaccine in sheep and goats was assessed by comparison with the positive and negative control groups. Daily clinical thermometry observation of the vaccinated animals was performed for 56 days PIV.

2.5. Assessment of vaccine immunogenicity

Blood serum samples (10 ml per Becton Dickinson Vacutainer tube) and whole blood (50 ml in tubes coated with EDTA/citrate) collected from sheep and goats were used to determine antigen-specific humoral (IgG, IgG2a, IgG1 antibodies by ELISA; in groups I and III) and T cell (stimulation index and IFN-γ production; in groups I–III) responses in all three animal groups at 28 and 56 days PIV.

2.6. ELISA

Ninety-six well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with pretitrated L7/L12 (2 μg/ml) and Omp16 (2 μg/ml) proteins in PBS, blocked for 1 h using PBS containing 1% ovalbumin (PBS-OVA; 200 μl/well), and washed with PBS containing 0.05% Tween-20 (PBS/Tw). Serial two-fold dilutions of the serum samples from the immunized sheep and goats (100 μl/well) were diluted in PBS-OVA, added to the plates and incubated for 1 h at room temperature. A donkey anti-ruminant IgG horseradish peroxidase conjugate (Sigma, St. Louis, MO, USA) and monoclonal antibodies specific for sheep IgG1 and IgG2 (Novus Biologicals, Littleton, CO, USA) were used for detection. After 90 min incubation at 37 °C and washing, specific reactivity was determined by addition of an enzyme substrate ABTS [2.2_azinobis (3-ethylbenzthiazolinesulfonic acid)] diammonium (Moss, Inc., Pasadena, CA, USA) 100 μl/well. The absorbance values were measured at 415 nm. The cut-off value for titer determination was calculated based on the mean OD value of wells containing only buffer (blank) ± three standard deviations.

2.7. Preparation of PBMC for lymphocyte proliferation assay

Peripheral blood mononuclear cells (PBMC) were enriched by density centrifugation using Ficoll-sodium diatrizoate gradient (DNA-Technology, Moscow, Russia) as previously described (Tabynov et al., 2016b). Cell number was adjusted to 10^7 viable cells per ml as determined by trypan blue dye exclusion method, and 50 μl of cell suspension (containing 5×10^5 cells) was added to triplicate flat-bottomed 96-well microtiter plates that had 100 μl of RPMI-1640 medium or RPMI-1640 medium containing 8.0 μg of purified *B. abortus* proteins L7/L12 and Omp16 per well or heat-inactivated (65 °C, 2 h) suspension of *B. melitensis* Rev.1 vaccine (concentration before inactivation 10^7 CFU/ml). The cell cultures were incubated for 7 days at 37 °C under 5% CO_2. After 7 days of incubation the cells were pulsed with 1.0 μCi of [^{3}H] thymidine per well and harvested onto glass filter mats and counted for radioactivity in a liquid scintillation counter. Cell proliferation results were converted to stimulation index (counts per minute [cpm] of wells containing antigens/cpm in the absence of antigens) for statistical comparison.

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