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Research Article

Safety of the novel vector vaccine against *Brucella abortus* based on recombinant influenza viruses expressing *Brucella* L7/L12 and OMP16 proteins, in cattle

Abstract

This paper presents the results of a study of the safety of new vector vaccine against *B. abortus* based on recombinant influenza A subtype H5N1 or H1N1 (viral constructs vaccine formulation) viruses expressing *Brucella* ribosomal protein L7/L12 and Omp16, in cattle. To increase the effectiveness of the vaccine, adjuvants such as Montanide Gel01 or chitosan were included in its composition. Immunization of cattle (5 animals per group) with the viral constructs vaccine formulation only, or its combination with adjuvants Montanide Gel01 or chitosan, were conducted via the conjunctival method using cross prime (influenza virus subtype H5N1) and booster (influenza virus subtype H1N1) vaccination schedules. Vaccine candidates were evaluated in comparison with the positive (*B. abortus S19*) and negative (PBS) controls. Comprehensive studies involving thermometry and clinical examination, hematology and biochemical blood analysis, showed that all of the viral constructs vaccine formulation, as well as their combination with adjuvants, compared to the commercial bacterial vaccine *B. abortus S19* were completely safe in cattle. Furthermore it is shown that the developed vaccines can effectively differentiate vaccinated animals from infected animals.

Introduction

Brucella abortus is a facultative intracellular pathogen capable of infecting and causing disease in both domestic animals and humans [1]. At present, brucellosis among cattle is prevented using live attenuated vaccines from the strains *B. abortus* 19 or RB51. These vaccines have a high immunogenic effectiveness, but have a number of serious disadvantages, primarily related to their ability to induce abortion in pregnant cows, secretion of the vaccine strain into the milk of vaccinated animals when they are used in adult cattle and the difficulty of differentiating between vaccinated animals and infected animals (only a concern for the *B. abortus* 19) [2]. Furthermore, both strains are pathogenic to humans [3]. Therefore, the development of an effective - and at the same time safe - vaccine against *B. abortus* is currently a problem.

In an effort to create an effective and safe vaccine against *B. abortus,* several research groups have developed subunit (recombinant proteins) [4-12], a DNA [13-18], or live vector vaccines (based on bacteria and viruses) [19-22]. All of these vaccines were safe when tested in animal models (laboratory mice), and some when tested in cattle. However, these vaccines remain inferior to commercial live attenuated vaccines in terms of protectiveness.

To solve this very significant problem we first proposed vector vaccine based on recombinant influenza viruses expressing the *Brucella* L7/L12 or Omp16 proteins. The influenza A virus contains a segmented genome consisting of eight negative-strand RNA fragments. Of these, the smallest fragment (NS) - encoding two proteins: viral nonstructural protein (NS1) and nuclear export protein (Nep) - is a convenient target for genetic manipulation as NS1 is able to tolerate foreign sequences exceeding its own length [25]. Thus, the ORF of NS1 was used for inserting *Brucella* sequences in this study. The A/Puerto Rico/8/34 (H1N1) strain was used as the backbone for obtaining influenza A virus vectors expressing *Brucella* L7/L12 or Omp16 sequences in the form of fusion proteins with the N-terminal 124 amino acid residues of NS1.

Our previous studies have shown that a bivalent vaccine formulation comprising a mixture of recombinant influenza A virus subtype H5N1 or H1N1 expressing the ribosomal L7/L12 or Omp16 proteins in prime and booster immunization mode (via conjunctival injection) in cattle induced a strong antigen-specific T-cell immune response, and most importantly provided a high level of protectiveness comparable to the commercial *B. abortus S19* vaccine and superior to the *B. abortus S19* vaccine in combination with Montanide Gel01 adjuvant [24]. Based on this, the next stage of our study was to evaluate the safety of the proposed new live vector vaccine in cattle. Additionally, we evaluated the possibility of differentiating infected animals from vaccinated animals using the developed vaccine.

Materials and Methods

Bacterial strains

The vaccine strain *B. abortus 19* (Shchelkovsky Biokombinat, Moscow oblast, Russia) and the virulent strain *B. abortus 544* (obtained from our institute's collection of microorganisms) were used in this study. The bacterial cells were cultured under aerobic conditions in tryptone soy agar (TSA; Sigma, St. Louis, MO, USA) at 37°C. All experiments with live Brucella were performed in biosafety level 3 facilities.

Generation of viruses

All viruses were generated as described previously [24]. Vaccine batches were produced in 10-day-old embryonated chicken eggs (CE; Lohmann Tierzucht GmbH, Cuxhaven, Germany) after three egg passages of viral constructs (Flu-NS1-124-L7/L12-H5N1, Flu-NS1-124-Omp16-H5N1, Flu-NS1-124-L7/L12-H1N1 и Flu-NS1-124-Omp16-H1N1).

Preparation of vaccines

Vaccine samples were prepared from the viral constructs Flu-NS1-124-L7/L12-H5N1, Flu-NS1-124-Omp16-H5N1, Flu-NS1-124-L7/L12-H1N1 and Flu-NS1-124-Omp16-H1N1, which accumulated in 10-day-old CE (Lohmann Tierzucht GmbH) at 34 °C for 48 h. The obtained allantoic suspensions of viral constructs with the same antigenic structure (H5N1 or H1N1) were combined in a single pool in a 1:1 ratio to obtain the bivalent vaccine formulation. Furthermore, the resulting mixtures of viral constructs (Flu-L7/L12-Omp16) were combined with adjuvants such as Montanide Gel01 (Flu-L7/L12-Omp16-MontanideGel01; Seppic, France) in a 80:20 ratio by volume (according to the manufacturer's recommendations) or chitosan oligosaccharide lactate (Flu-L7/L12-Omp16-chitosan; Sigma-Aldrich) in a final concentration of 0.05%, and the mixtures were stirred using a magnetic stirrer for 5-7 min.

Cattle and bioethics

Used 25 head of cattle (heifers), Kazakh white breed (meat direction) aged 1-1.5 years-old. All animals were seronegative for B. abortus, which was confirmed by analysis of blood serum using the Rose Bengal test (RBT; Antigen, Almaty, Kazakhstan), serum agglutination test (SAT; Microgen, Moscow, Russia), complement fixation test (CFT; Microgen) and enzyme-linked immunosorbent assays (ELISA; Brucella-Ab C-ELISA, Svanova Biotech AB, Sweden) according to the manufacturers' instructions. Heifers were divided into five groups (5 animals per group): three experimental groups vaccinated with Flu-L7/L12-Omp16, Flu-L7/L12-Omp16-MontanideGel01 or Flu-L7/L12-Omp16-chitosan, one negative control group (PBS), and one positive control (B. abortus S19) group. Each group of animals was kept in a separate room and had free access to water and feed throughout the experiment.

This study was carried out in compliance with 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: 0513/107).

Vaccination

Cattle in the experimental groups were immunized twice via the conjunctival route of administration at an interval of 30 days with vaccines generated from the viral vector subtypes H5N1 (prime vaccination) and H1N1 (booster vaccination). The detailed animal immunization scheme is shown in Table 1. Cattle in the positive control group were immunized once subcutaneously in the neck region (right side) with a commercial vaccine B. abortus S19 (Shchelkovsky Biokombinat, Russia) at a dose of 80 x 10^9 CFU/animal (according to the manufacturer's instructions). Cattle in the negative control group were administered subcutaneously with 2.0 ml of PBS.

Safety assessment of vaccines

The safety of the vaccines generated from the viral constructs was determined and compared with the negative (PBS) and positive (B. abortus S19) control groups. The vaccinated cattle were clinically observed daily by thermometry for 60 days after the initial vaccination (IV). Furthermore, blood samples were taken on days 0, 7, 14, 30, 37, 44, 60 post-IV from the jugular vein (serum and whole blood using Vacutainer tubes; Becton Dickinson, USA) for hematological and biochemical studies.

Hematological and biochemical blood tests

For hematological analysis, whole blood samples were assayed using the 540 T-Coulter Counter (Coulter Electronics, Hialeah, FL, USA) and ADVIA120 Hematology System (Bayer Healthcare LLC, Tarrytown, NY, USA) automatic blood analyzers; the following parameters were determined: hemoglobin concentration, hematocrit, red blood cells, white blood cells, platelets, stab and segmented neutrophils, eosinophils, lymphocytes, and monocytes.

Biochemical studies of the serum samples were performed on a VITALAB Selectra 2 automated analyzer (Merck, Germany) using commercial kits from DiaSys Diagnostic Systems GmbH (Germany); the following parameters were determined: total bilirubin, direct bilirubin, creatinine, cholesterol, total protein, urea, glucose, aspartate aminotransferase, and alanine aminotransferase.

Differentiation of infected from vaccinated animals

In order to differentiate of infected from vaccinated animals, blood samples were collected from cattle on days 0, 7, 14, 30, 37, 44, 60 post-IV, and 7, 14, 21 and 30 days after post-challenge for analysis by the RBT (Antigen), SAT (Microgen) and CFT (Microgen) according to the instructions included with the kits. On day 60 post-IV, cattle

Table 1: The immunization schedule of cattle (heifers) with viral constructs vaccine formulations.

Vaccine*	Number of animals	Dose prime vaccination (H5N1), $\log_{10}$ EID_{50} / animal	Dose booster vaccination (H1N1), $\log_{10}$ EID_{50} / animal
Flu-L7/L12-Omp16	5	8.74+8.74	8.5+8.25
Flu-L7/L12-Omp16-Montanide Gel01	5	8.64+8.64	8.4+8.15
Flu-L7/L12-Omp16-chitosan	5	8.43+8.43	8.2+7.95

* Volume vaccine for cattle by conjunctival method of administration was 1 ml (0.5 ml to each eye)

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from the experimental, negative (PBS) and positive (*B. abortus S19*) control groups were subcutaneously challenged with a virulent strain of *B. abortus 544* at a dose of 5 x 10^8 CFU/animal.

Statistical analysis

We counted the mean and standard deviation of rectal temperature, hematological and biochemical parameters in groups of cattle. These values were compared with the normal physiological values.

Results

1 Safety assessment of the vaccines in cattle

Clinical observations with thermometry: This study showed that immunization of animals with viral constructs vaccine formulation only, or its combination with adjuvants did not have any negative impact on the overall clinical status (behavior, appetite, etc.) of the animals throughout the observation period. The body temperature of the animals in the experimental groups was within normal limits during the observation period (Figure 1). No side effects (expiration, conjunctivitis, etc.) were observed at the site of conjunctival administration.

In the positive control group vaccinated with *B. abortus S19*, no animals showed any signs of any disease or changes in behavior or appetite during the period of clinical observation, similarly to the animals in the negative control group (PBS). However, the animals in the positive control group displayed an increase of body temperature up to 40.9°C for 1-3 days after vaccination (Figure 1). Furthermore, infiltrates up to 7 cm in diameter formed at the site of subcutaneous vaccination, which were completely resorbed by 14 days after vaccination.

Hematologic and biochemical studies: Hematological and biochemical analysis revealed that all of the studied parameters remained consistent with normal physiological ranges [25, 26] during the entire period of observation in all groups, and in spite of dynamic changes, did not exceed the limits of the normal ranges (Figures 2 and 3). The only exception occurred in the group of animals vaccinated with the Flu-L7/L12-Omp16-MontanideGel01 and B. abortus S19 vaccines, in which band neutrophils were detected at 7 days after vaccination; according to Gromyko [27], band neutrophils are associated with a slight infectious process (in this case, with vaccination).

Differentiation of vaccinated and infected animals

Blood samples were collected from the cattle on days 0, 7, 14, 30, 37, 44, 60 post-IV and also 7, 14, 21 and 30 days after challenge with *B. abortus 544* for serologic screening tests, such as the RBT, SAT and CFT. Additionally, the serum of cattle vaccinated with *B. abortus* 19 was analyzed at the same time points after vaccination. No antibodies were detected in cattle vaccinated with Flu-L7/L12-Omp16, Flu-L7/L12-Omp16-MontanideGel01 or Flu-L7/L12-Omp16-chitosan in the

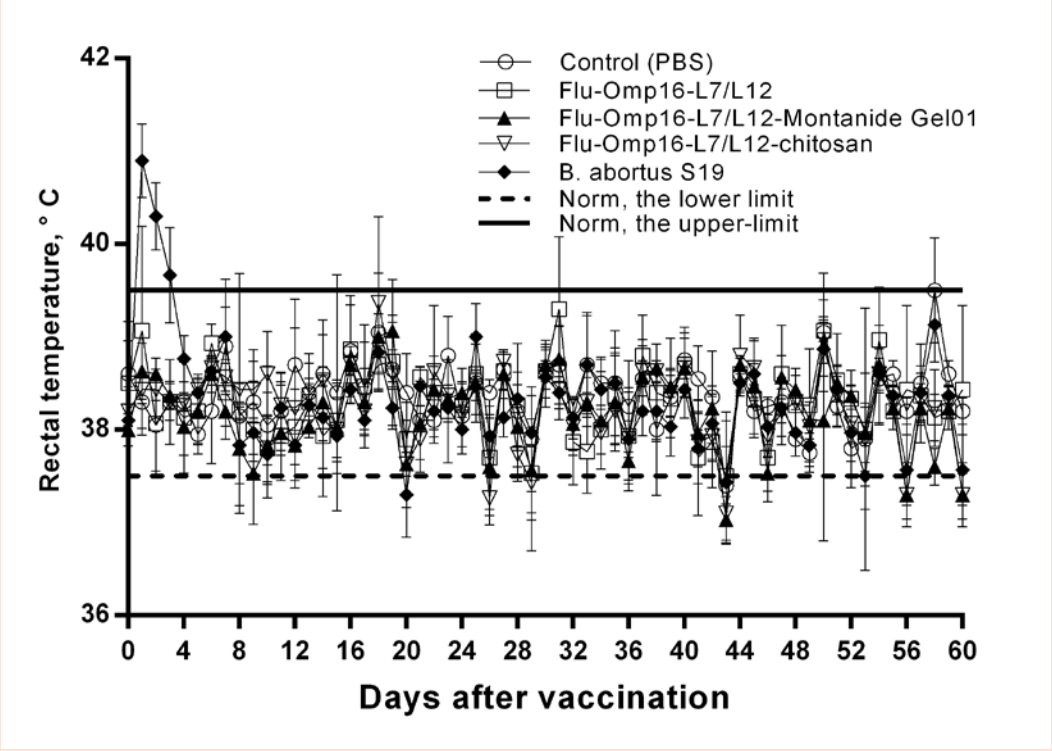

Figure 1: Rectal temperature in cattle after vaccination. Vaccination of cattle was carried out twice with an interval of 30 days with viral constructs vaccine formulation only (Flu-L7/L12-Omp16) or a combination thereof with adjuvants (Flu-L7/L12-Omp16-Montanide Gel01 or Flu-L7/L12-Omp16-chitosan) or a single with commercial vaccine *B. abortus S19*. The animals of the negative control group as an inoculum were administered with PBS. The data are presented as mean ± standard deviation (SD).

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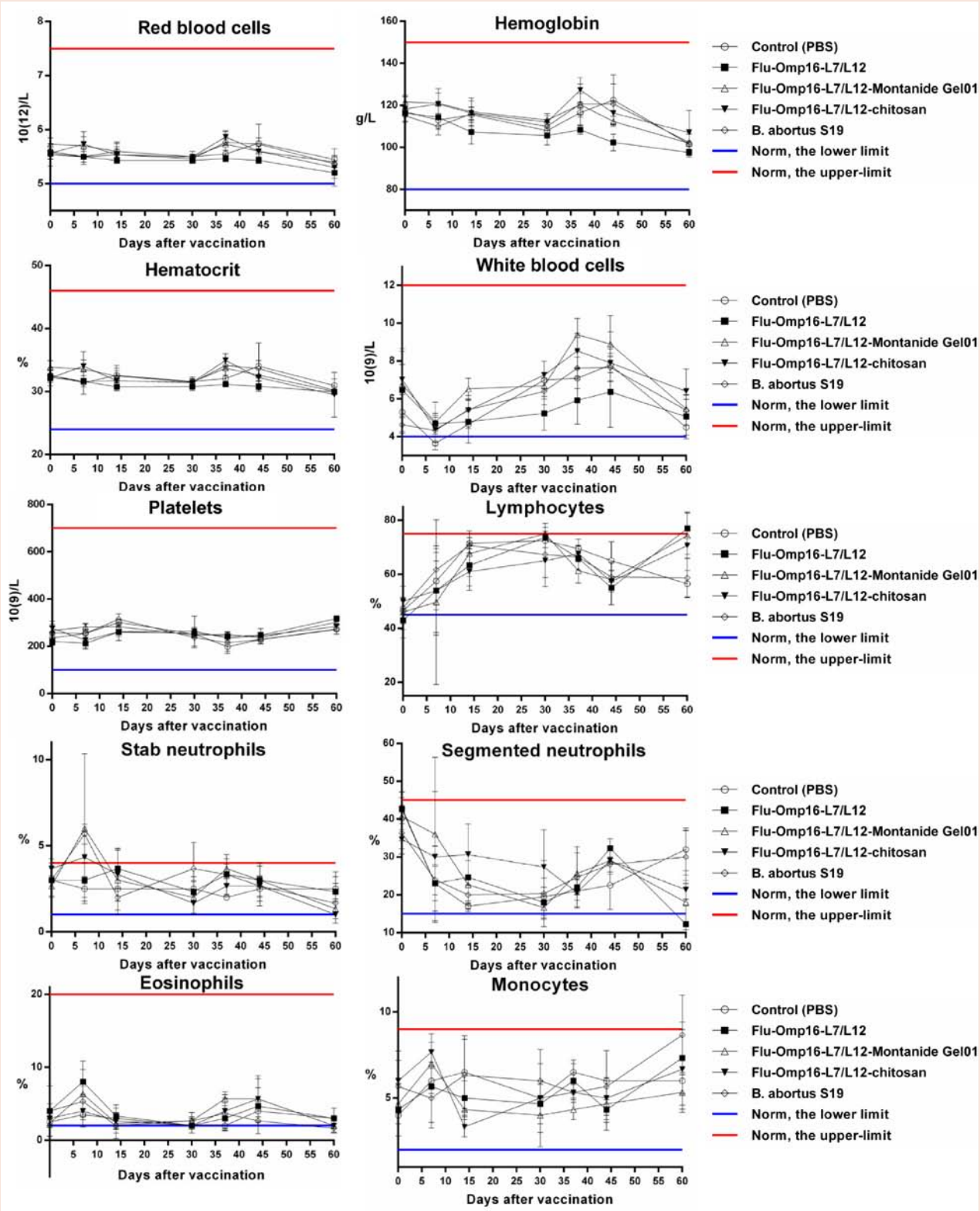

Figure 2: Hematological studies of cattle whole blood samples on days 0, 7, 14, 30, 37, 44, 60 after the initial vaccination. Vaccination of cattle was carried out twice with an interval of 30 days with viral constructs vaccine formulation only (Flu-L7/L12-Omp16) or a combination thereof with adjuvants (Flu-L7/L12-Omp16-Montanide Gel01 or Flu-L7/L12-Omp16-chitosan) or a single with commercial vaccine *B. abortus S19*. The animals of the negative control group as an inoculum were administered with PBS. The data are presented as mean ± standard deviation (SD).

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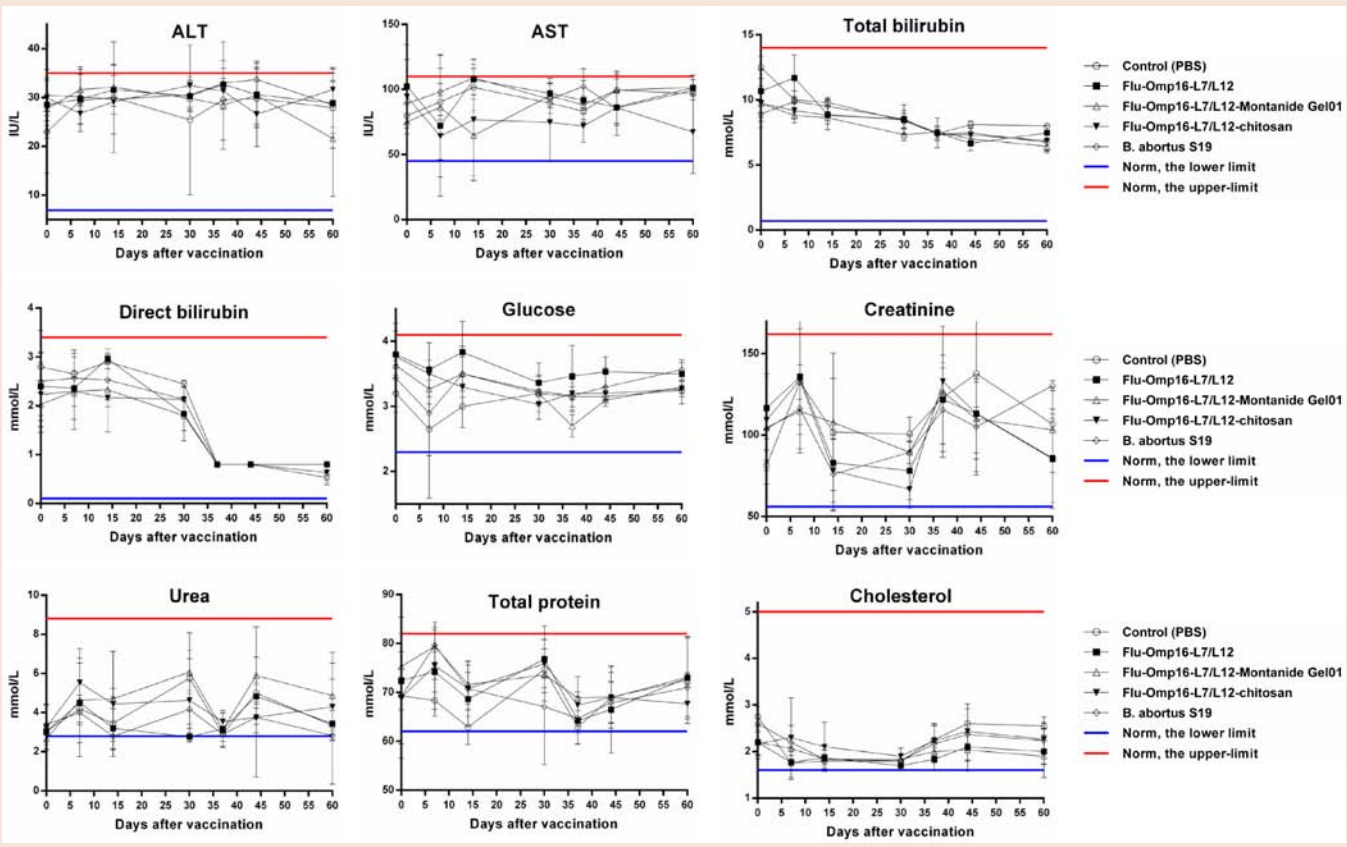

Figure 3: Biochemical studies of cattle blood serum samples on days 0, 7, 14, 30, 37, 44, 60 after the initial vaccination. Vaccination of cattle was carried out twice with an interval of 30 days with viral constructs vaccine formulation only (Flu-L7/L12-Omp16) or a combination thereof with adjuvants (Flu-L7/L12-Omp16-Montanide Gel01 or Flu-L7/L12-Omp16-chitosan) or a single with commercial vaccine *B. abortus S19*. The animals of the negative control group as an inoculum were administered with PBS. The data are presented as mean ± standard deviation (SD).

test periods after prime and boost vaccination using the RBT, SAT or CFT. However, as expected, in the group of cattle vaccinated with *B. abortus* 19, antibodies were detected using the RBT, SAT and CFT at 7 and 30 days after vaccination, respectively (data not shown). After challenge of the cattle vaccinated with Flu-L7/L12-Omp16, Flu-L7/L12-Omp16-MontanideGel01 or Flu-L7/L12-Omp16-chitosan (data not shown), in most animals antibodies were initially detected using the RBT (primarily) at 14 days, and were detected using the SAT and CFT by 30 days.

Discussion

The present work is a continuation of a series of studies aimed at developing a safe and effective vaccine against *B. abortus*. As already pointed out to solve the problem of specific prophylaxis *B. abortus* we was first proposed vector vaccine based on recombinant influenza viruses expressing *Brucella* L7/L12 or Omp16 proteins. Our previous studies have shown that this vaccine in cattle induced a strong antigen-specific T-cell immune response, and most importantly provided a high level of protectiveness comparable to the commercial *B. abortus S19* vaccine and superior to the *B. abortus S19* vaccine in combination with Montanide Gel01 adjuvant [24]. Safety data of developed vaccines, as well as its ability to differentiate infected animals from vaccinated animals have never been described and presented for the first time in this paper.

Inclusion of adjuvants in the vaccine was due to the need to enhance its effectiveness. In view of the conjunctival route of vaccine administration, we focused on commercial adjuvants such as Montanide Gel01 and chitosan, which according to the manufacturer's recommendations and in some publications [28-31] can be incorporated into vaccines with a mucosal route of administration.

In previous studies, it was shown that as the size of the *NS1* gene decreased in viral vectors, the degree of attenuation of the influenza A viruses increased [32]; however, it is well known that attenuation of influenza viruses may be dependent on the properties of the foreign insert in the C-terminal part of the trancated *NS1* protein [33]. Therefore, we considered it necessary to study the safety or degree of attenuation of the constructed recombinant influenza A viruses in cattle. It should be noted that in these studies, along with the traditional methods used for assessing the safety of veterinary vaccines, we also employed more sensitive methods such as hematological and biochemical blood analysis, which enable the early detection of any disease with unclear clinical symptoms in the body. Thus, as a result of our comprehensive studies involving clinical examination

with thermometry, hematology and biochemical blood analysis, it was found that all of the viral constructs vaccine formulation, alone or in combination with adjuvants, were completely safe for cattle in prime and booster immunization mode compared to the commercial *B. abortus S19* vaccine. In our previous studies we demonstrated the replication-deficient properties of the viral vectors and confirmed the absence of viral transmission from vaccinated to non-vaccinated animals [34]. Interestingly, one vaccine vector is based on the prepandemic flu A/H5N1 delNS1 vaccine. It was previously shown that this vaccine is completely safe and immunogenic when tested in a variety of laboratory models (chickens, ferrets and rhesus macaques) [35] and humans [36]. Therefore, we assume that this vaccine could not only be used for cattle but also for humans in the future.

The final stage of this study investigated whether it was possible to distinguish between vaccinated and infected animals when the recombinant influenza A viruses expressing the *Brucella* proteins L7/L12 and Omp16 were used as vaccines. The use of traditional brucellosis vaccines is significantly complicated by the difficulty of differentiating between vaccinated and infected animals due to the presence of an O chain in the *Brucella* lipopolysaccharide responsible for the agglutinogenic properties of serum [2]. The recombinant influenza A viruses we constructed do not contain this factor; therefore, as expected, and in contrast to the positive control group of animals (*B. abortus* 19), no antibodies were detected using the RBT, SAT or CFT in cattle serum during the entire observation period after prime and boost vaccination with Flu-L7/L12-Omp16, Flu-L7/L12-Omp16-MontanideGel01 or Flu-L7/L12-Omp16-chitosan. After challenge of the cattle vaccinated with viral construct vaccine formulations, in most animals antibodies were initially detected using the RBT (primarily) at 14 days, and were detected using the SAT and CFT by 30 days. In the groups of animals immunized with *B. abortus* 19 antibodies could be detected using the RBT, SAT and CFT at 7 days post-challenge. These data show that after immunization with Flu-L7/L12-Omp16, Flu-L7/L12-Omp16-MontanideGel01 or Flu-L7/L12-Omp16-chitosan, it is possible to effectively differentiate between vaccinated animals and infected animals.

Thus, we can conclude that our proposed new candidate vaccine against *B. abortus* - bivalent vaccine formulation consisting of a mixture of recombinant influenza A viruses subtypes H5N1 or H1N1 expressing *Brucella* ribosomal protein L7/L12 or Omp16 in prime and booster immunization mode (with conjunctival injection) completely safe for cattle, furthermore it is can effectively differentiate infected from vaccinated animals. Based on the data, as well as previously published results [24], for practical use in cattle we recommended bivalent vaccine formulation containing the adjuvant Montanide Gel01.

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