Vaccine 32 (2014) 1877–1881

Contents lists available at ScienceDirect

Vaccine

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

Evaluation of the immunogenicity and safety of *Brucella melitensis* B115 vaccination in pregnant sheep

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ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form 14 January 2014
Accepted 21 January 2014
Available online 7 February 2014

Keywords:
Brucella melitensis
B115 strain
Vaccination
Sheep
Pregnancy
Safety

ABSTRACT

In spite of its limitations, Rev.1 is currently recognized as the most suitable vaccine against *Brucella melitensis* (the causative agent of ovine and caprine brucellosis). However, its use is limited to young animals when test-and-slaughter programs are in place because of the occurrence of false positive-reactions due to Rev.1 vaccination. The *B. melitensis* B115 rough strain has demonstrated its efficacy against *B. melitensis* virulent strains in the mouse model, but there is a lack of information regarding its potential use in small ruminants for brucellosis control. Here, the safety and immune response elicited by B115 strain inoculation were evaluated in pregnant ewes vaccinated at their midpregnancy. Vaccinated ($n=8$) and non-vaccinated ($n=3$) sheep were periodically sampled and analyzed for the 108 days following inoculations using tests designed for the detection of the response elicited by the B115 strain and routine serological tests for brucellosis [Rose Bengal Test (RBT), Complement Fixation Test (CFT) and blocking ELISA (ELISAb)]. Five out of the 8 vaccinated animals aborted, indicating a significant abortifacient effect of B115 inoculation at midpregnancy. In addition, a smooth strain was recovered from one vaccinated animal, suggesting the occurrence of an in vivo reversion phenomenon. Only one animal was positive in both RBT and CFT simultaneously (91 days after vaccination) confirming the lack of induction of cross-reacting antibody responses interfering with routine brucellosis diagnostic tests in most B115-vaccinated animals.

1. Introduction

Ruminants are considered the main source of infection of *Brucella* spp. for humans [1]. *Brucella melitensis*, the main etiologic agent of small ruminant brucellosis (SRB), is endemic in most Mediterranean countries, Latin America, Middle East and Central Asia [2] and it is recognized as the most important causative agent of human brucellosis in most parts of the world [3]. In nearly all developed countries in which the disease was present, the control/eradication of SRB has been achieved through three main strategies: hygienic measures, test-and-slaughter policy and/or Rev.1 vaccination. *B. melitensis* Rev.1 [4] has been widely recognized as the best vaccine against *B. melitensis* currently available for small ruminants, and many studies have demonstrated its usefulness in different conditions [5–8]. However, certain drawbacks linked to the smooth nature of Rev.1 have limited its application in the field as occurs with *Brucella abortus* S19 vaccine in cattle. The use of Rev.1 is usually restricted to prepuberal ewes and goats due to its residual virulence (that may lead to abortion in pregnant animals [9] and disease in humans [10]) and the induction of diagnostic interferences on the serological tests used for diagnosis of SRB (due to the elicitation of anti-S-LPS antibodies) [11]. In the case of bovine brucellosis the RB51 strain (a *B. abortus* rough strain) is an alternative to S19 vaccination that may overcome at least in part some of these drawbacks, and whose use has proved successful in several countries worldwide including Portugal, Spain and Argentina [12–14]. However, the protection induced by RB51 in

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0264-410X/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2014.01.070

pregnant sheep against *B. melitensis* is very limited compared to that evoked by the Rev.1 vaccine [15]. *Brucella melitensis* B115 strain (herein after B115), a natural rough stable strain [16], has demonstrated its usefulness for the immunization not only against *B. melitensis*, but also against *B. abortus* and *B. ovis* in the mouse model [17,18]. However, only one study from 1960 assessed its efficacy compared to Rev.1 in non-pregnant goats [19], although the use of animals in poor conditions make difficult the extrapolation of the results obtained. Considering these promising results and the lack of an alternative to the Rev.1 vaccine for the immunization against *B. melitensis* in small ruminants, the present study was carried out aiming to (i) assess the safety of B115 strain in pregnant ewes vaccinated at midpregnancy (around 90 days of pregnancy), (ii) evaluate the risk in vaccinated animals of B115-shedding, that could lead to horizontal transmission and (iii) assess the serological and cellular immune response induced in vaccinated and non-vaccinated animals using tests designed for the detection of the response elicited by the B115 strain and routine serological tests for SRB [Rose Bengal Test (RBT), Complement Fixation Test (CFT) and blocking ELISA (ELISAb)].

2. Materials and methods

2.1. Experimental design and vaccination

Eleven Manchega-breed ewes aged approximately 12 months from an officially brucellosis free flock [according to European (91/68/EEC) and national regulations (Spanish Royal Decree 1047/2003)] were randomly selected. All ewes were seronegative for brucellosis and other abortifacient infectious diseases (Q fever, toxoplasmosis, chlamydiosis, visna-maedi, and border disease). They were mated after oestrus synchronization and randomly divided into two experimental groups: (i) vaccinated group (VG; $n = 8$) and (ii) control animals (non-vaccinated group, NVG; $n = 3$). All animals were kept together in the same isolated pen with food and water provided ad libitum. The VG was vaccinated subcutaneously in the axillary region with $1–2 \times 10^9$ CFU of B115 in a volume of 1 mL in the last third of pregnancy (around 90 days of gestation). Previously, the freeze-dried B115 vials were reconstituted in 7.6 mL of sterile saline solution before inoculation. The B115 viable bacteria concentration of each vial used in the present study was verified by counting plate on the day of vaccination. All husbandry practice and animal procedures were authorized by the animal research committee from Madrid Region (10/230335.9/11).

2.2. Sampling

The B115 bacteremia in all animals was monitored at 3, 7, 14 and 28 days post-vaccination as well as the day of abortion/parturition and 7, 14 and 21 days after the reproductive outcome. Sera samples from each animal (VG and NVG) were collected at 0 and 3 days post-vaccination (d.p.v.), and thereafter weekly until slaughter (108 d.p.v.). Whole blood for specific interferon-gamma (IFN-γ) detection was taken from all animals on the day of vaccination (day 0; before immunization) and 7, 14, 28 and 63 days post-vaccination (d.p.v.) Thereafter, whole blood samples were collected weekly until 99 days post-vaccination. Milk and vaginal swabs were collected from all animals the day of parturition/abortion and 7, 14 and 21 days after the reproductive event. A subset of animals was also sampled for milk ($n = 5$) and vaginal swabs ($n = 6$) at 28 and 35 days, respectively, after parturition/abortion. Samples from spleen, lung, liver and/or stomach content were collected from fetuses and subjected to bacteriological analysis on the same day or refrigerated at 4 °C and processed the following day. Similarly, samples from all ewes (liver and mammary lymph nodes) and viable lambs (liver and spleen) were collected at the end of the experiment (108 days post-vaccination and 4–6 weeks after abortion/parturition). These samples were stored at −20 °C until further analysis.

2.3. Serology

Serum samples were subjected to classical brucellosis serological tests based on smooth LPS antigens of *Brucella* [RBT, CFT and ELISAb]. RBT and CFT were carried out according to Alton et al. [20]. The Blocking ELISA (INGEZIM BRUCELLA COMPAC, Ingenasa, Tres Cantos, Spain) was performed according to manufacturer instructions. A specific B115-CFT based on the detection of antibodies against B115-rough antigen was performed as previously described by Adone et al. [21].

2.4. Cell mediated immune response

Whole blood samples were analyzed to determine the specific IFN-γ production after in vitro antigen stimulation as previously described by Duran-Ferrer et al. [29] with slight modifications. Whole blood was processed within the first 6 h after collection. Each blood sample was divided into three aliquots of 1 mL: aliquot 1 was stimulated with 10 μL of a cell suspension of *B. abortus* S99 prepared for complement fixation test antigen [20] but 10 times more concentrated, aliquot 2 was inoculated with 40 μL of Brucellergen (Symbiotics OCB) and aliquot 3 was stimulated with 50 μL of PBS (Phosphate Buffered Saline). All samples were incubated at 37 °C in a humidified atmosphere for 20 ± 2 h and plasma was then recovered after centrifugation at 490 g for 15 min. All samples were stored at −40 °C until being analyzed with the Bovigam ELISA (Prionics, Schlieren-Zurich, Switzerland) according to the manufacturer instruction. Quantitative results [ODs after stimulation with PBS (OD_{PBS}), S99 antigen (OD_{S99}) and B115 antigen (OD_{B115})] and the stimulation index $SI = OD_{ag}/OD_{PBS}$ [22] were recorded in an Excel file. The threshold (considering animals positive if $SI \geq 2.5$) previously described [22] was used.

2.5. Bacteriology and molecular analysis

Bacteriology was performed according to the OIE guidelines [23] and Alton et al. [20]. Briefly, approximately 2 g from each sample was placed in 1.5 mL of sterile PBS, macerated and cultured in Farrel selective medium. Plates were incubated at 37 °C for at least 14 days. Milk and vaginal swabs were directly cultured in Farrel medium. A subset of milk samples ($n = 30$) were centrifuged at $490 \times g$ for 28 min. Milk swabs recovered from the upper layer after centrifugation were directly plated in Farrel medium. Finally, a total of 10 mL of whole blood was seeded in a biphasic Castañeda medium and incubated at 37 °C at least 30 days.

All *Brucella*-like colonies were suspended in 200 μL of sterile water and heat-inactivated at 100 °C for 15 min. Molecular identification was performed by a *Brucella*-genus specific PCR [24]. Phenotype (rough or smooth) of all *Brucella* isolates was assessed by crystal violet dye and Acriflavine test according to Alton et al. [20]. PCR and sequencing analysis of gene *wzm* were performed in all isolates for B115 strain identification according to Adone et al. [17]. In addition, PCR and sequencing analysis of genes *manC*$_{oag}$, *manCore* and *wboA* (also involved in LPS synthesis) were performed in *B. melitensis* isolates, showing smooth phenotype, recovered from ewe 32544. Isolates from ewe 33806 and 32544 were further typed using Multiple Loci VNTR Analysis (MLVA) [25].