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Preliminary results of sero-conversion of kids and lambs vaccinated with *Brucella melitensis* Rev -1 strain. Current achievements and feature challenges on brucellosis

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Abstract

Sheep and goat brucellosis is an endemic and most important infectious disease of livestock in Albania. It continues to remain a frequent zoonotic disease and an important public health issue. Among available strategies, mass vaccination is an acceptable, cost effective approach, and is a widely used strategy in many countries including some neighbouring Balkan countries. Albanian veterinary services supported by the European Union-funded PAZA project (Protection Against Zoonotic diseases, Albania) applied two successive annual mass vaccination campaigns that aimed to vaccinate all small ruminants in the country. These two campaigns aimed at significantly reducing disease spread, however, a small number of infection foci could remain and persist in some parts of country. Post-vaccination surveillance is essential for early detection and proper control of cases of brucellosis that might re-emerge. Limitation major complication arising from mass vaccination is the difficulty of interpretation of the results of serological tests conducted to diagnose the disease.

The aim of this study was to evaluate the proportion of vaccinated animals that showed sero-conversion and the duration of detectable levels of agglutinins (antibody) against brucellosis in vaccinated animals.

Methods. In total, 69 individual animals, 23 lambs and 46 kids aged from 4 to 7 months, were sampled at monthly intervals. Jugular blood was collected before vaccination and at intervals thereafter and tested by means of the Rose Bengal test. All animals were serologically negative before vaccination with modified live *Brucella melitensis* Rev.1 strain vaccine. Rose Bengal test was performed before vaccination, 18 days, 2, 3 and 4 months after vaccination.

Results. Eighteen days after vaccination, 63 out of 69 animals (91.3%) 82.6% of lambs (19 out of 23 lambs) and 95.6% of goat kids (44 out of 46) showed strong sero-conversion in Rose Bengal test. The proportion of positive vaccinated animals decrease progressively over time, and 4 months after vaccination all lambs were sero-negative; only one kid remain weakly sero-positive in RB test.

Conclusion. Sero-conversion rate in young small ruminants, vaccinated against *Brucella melitensis* was within protective herd immunity limits. RB test could be used, with high confidence, for brucellosis surveillance four months after vaccination with *Brucella melitensis* Rev.1 strain vaccine administered intraconjunctivally in animal between 3 and 7 months of age.

Key words: Zoonoses, vaccination, disease control, surveillance, agglutinins, *Brucella melitensis*

Introduction

Brucellosis is an important bacterial infectious disease due by *Brucella* species, described first in 1850 in Malta. *Brucella* spp. are Gram negative bacteria that affect both animals and humans. The medical and veterinary important pathogenic Brucella are *Brucella abortus, B. melitensis, B.suis, B.ovis, B.neotomae, B. canis, B.ceti, B. pinnipedialis, B.microti, B.inopinata* [8, 12, 17, 18]. Among all veterinary important bacteria, *Brucella* genome is unusually, it is composed of two chromosome, except *Brucella suis* biovar 3. *Brucella* spp. are well adapted with specific host, however transmission between speccies is possible. *Brucella abortus, B. melitensis, B.suis* have several biovars, which could be used for identifying and tracing the source of infection during disease outbreaks [2, 8, 18]. It is accepted that principal host serve as reservoir for *Brucella* type. As general rule applying strategic control measures for *Brucella* controlling in natural host is adequate for disease control in other species, including humans. Availability of modern molecular techniques overpass discrimination's power limitations of phenotypic methods allowed discovering novel *Brucella* species and *Brucella* like organisms [17]. Based on molecular methods,

brucellae species are highly homogenous and recently is proposed that all species are serovar of *Brucella melitensis* [8, 18]. The classification of *Brucella* species is an ongoing process, and based on recent data it is clear that we are at beginning of understanding the all species that are able to carry the range of *Brucella* spp and *Brucella* like organisms and their pathogenic aspects, route of transmission, mechanisms of persistence, etc. [17]. Brucellosis is an important highly contagious zoonotic disease. Human can be affected by all *Brucella species,* except *B.ovis* and non-pathogenic *B.neotomae* [8].

Historically, Brucellosis is known with different names according, place, authors, species affected and limitation of knowledge's. Human Disease is known as Malta Fever, Undulant Fever, Mediterranean Fever, Rock Fever of Gibraltar, Gastric Fever and in animals is known Bang's Disease, Enzootic Abortion, Epizootic Abortion, Slinking of Calves, Ram Epididymitis, Contagious Abortion [8, 12, 18, 20]. In humans, brucellosis is an very important zoonotic disease. The humans get infection by consuming of unpasteurized milk, uncooked meat, close contact with infected animals, especially birth fluids, foetal membranes, placentas [1, 3, 5, 6, 10, 11, 12, 17,]. In addition, humans could be exposed in laboratories and during applying the live modified *Brucella* spp. vaccines. Human - human transmission is rare, but possible either through sexual contact or from mother to child. In Albania, *B.melitensis* and *B.abortus* are important, in particular *B.melitensis* is most prevalent [1, 5]. However, *B.suis* could be a threat as far as there are some report that swine Brucellosis circulate in wild boar and domestic pigs in Montenegro, Serbia, Croatia [9].

Study design and its justification

Vaccination strategy have been used for almost 60 years, however there are some limitations of vaccine based on smooth colonies such as *B.melitensis* Rev 1: ability to induce abortion in pregnant animal; the potential risk for human infection; limited efficacy in other natural species; limited ability to prevent infection; seraconversion after exposure, and official serological test do not discriminate antibodies from infection and those after vaccination [15]. In total 69 young animals (23 lambs and 46 kids) age 3-7 months were vaccinated with a Modified Live Vaccine, *Brucella melitensis* Rev 1 strain, applied intra-conjunctively and Rose Bengal Test (RBT) was performed, at day zero (prevaccination), and approximately monthly after vaccination.The questions were; could we use Rose Bengal method as screening test and other serological tests in vaccinated animals to investigate suspected cases of brucellosis?; how long the agglutinins against *B.melitensis* Rev 1 persist in vaccinated replacement animals? The aims of study were: a) to evaluate the proportion of vaccinated animals that showed sero-conversion and b) duration of detectable levels of agglutinins (antibody) against brucellosis in vaccinated animals.

Materials

The blood collection procedure in lambs and kids was performed by experienced veterinarians following proper physical restraint of animals to ensure both personel and animal safety. Initially, a total 69 blood samples were collected, number of animals in second bleeding was the same and in last bleeding was 55 animals. Serum was harvested and preserved at -20^{0}C until the RBT was performed. The animals species at day zero were 23 lambs and 46 kids aged 4 to 7 months. Modified live *Brucella melitensis* Rev.1 strain vaccine and Rose Bengal Test were provide by PAZA project.

Methods:

Rose Bengal Test was performed at day 0, 18, 60, 90, 120. Positive and negative control and *Brucella* antigen stained with haematoxylin were available at infectious disease laboratory at Faculty of Veterinary Medicine, Tirana Albania. The test procedure was 30 µL of serum samples was mixed with an equal volume of RBT antigen, using a toothpick. Both sera and RB were equilibrated at room temperature and shaken to re-suspend any bacterial sediment. The reaction was read after 4 minutes and positive results were marked with 1, 2, 3, 4 pluses (data not shown) depended on intensity of agglutinations. The data were analysed by using Excel Data Analyzes.

Results

Table 1 and Figure 1 represent the results of study. All animals at day 0 (before vaccination) tested negative in RBT. Eighteen days after vaccination, 63 out of 69 subjects shown strong sero-conversion in Rose Bengal Test. The number of positive animals declined at monthly interval and at day 120 (four months) after vaccination all lambs were negative and only a kid remain weakly positive in RBT.

Table 2 Results of Rose Bengal test at intervals performed

Day of performed RB test	*Lambs*			*Kids*			*Lambs&Kids*		
	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
0	**0**	23	23	**0**	46	46	**0**	69	69
18	**19**	4	23	**44**	2	46	**63**	6	69
60	**15**	6	21	**28**	15	43	**43**	21	64
90	**7**	11	18	**13**	24	37	**20**	35	55
120	**0**	19	18	**1**	36	37	**1**	54	55

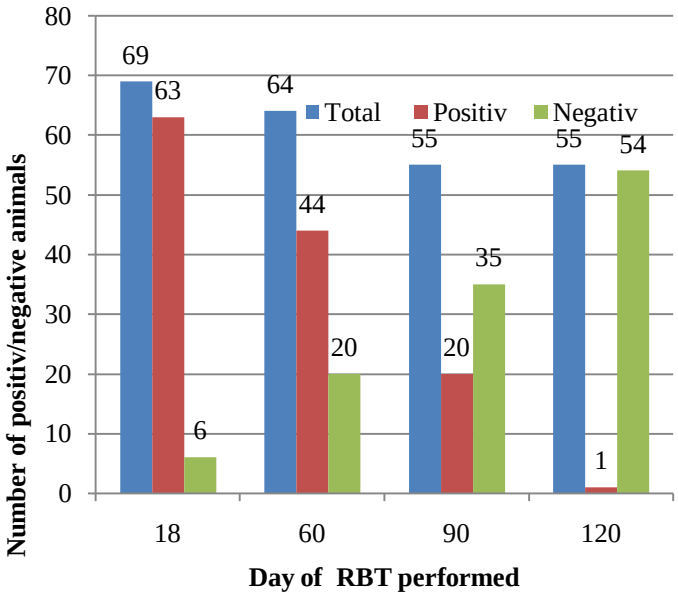

Figure 2: Results of Rose Bengal Test performed after vaccination

Discusion

Vaccination against brucellosis remain a strategy of choice for successful control of brucellosis in conditions similar to Albanian. *B. melitensis* strain Rev.1 vaccine is only vaccine recommended for sheep and goats brucellosis [3, 4, 7, 12, 14, 15, 18]. The *B. melitensis* strain Rev.1 vaccine has also been shown to protect sheep against *B. ovis* [15]. *B. melitensis* strain Rev.1 vaccine induce serological titres on diagnostic tests, including RBT. As a general rule, *B. melitensis* strain Rev.1 vaccine must be applied on healthy non-pregnant adult sheep and goats, and in lambs and kids 3-8 months [3, 15].

In best authors knowledge, mass vaccination strategy was suitable strategy for Albania and is recommended when the prevalence of disease is high, animal identification and animal movement control is not in place, veterinary services performance is not adequate, etc. Immunity to brucellosis is related with cell mediated immunity and humoral immunity. Sero-conversion rate in young small ruminants, vaccinated against *Brucella melitensis* was 91.3% and very strong. This results was within protective herd immunity limits [13, 15]. In first testing after vaccination, 8.77% of vaccinated animals tested negative on RBT. This could be explained in part because *Brucella, c*ompared to other Gram-negative bacteria, induces a reduced innate immune response, and a lower rate of maturation and activation of dendritic cells [3, 12]. Lack of stimulation of innate immune responses by natural infection with *Brucella,* or after vaccination with attenuated strains, may impair the development of robust adaptive immune responses. In addition this feature partially could explained some serological negative results of infected animals as well. After host exposure to *Brucella* spp, either M cells (when port of entry is digestive route) in Payer's patch and dendritic cells in other port of infections, are first involved in infection. Immune responses against *B. melitensis* strain Rev.1 vaccine include role of MHC II, CD4+, CD8+, γδ T-cells (typically in ruminant animals circulate large number of subsets γδ cells), Th1, Th2, Th17 and Th17-reg cells. The role of Th17 and Treg cells in *Brucella* infection or protective immunity after vaccination is not well known, however, Treg cells, play a crucial role in regulating immune responses, by involvement of CD4+ cells which suppress Th1, Th2 and Th17 responses. This could explain missing of lesions at port of entry [17, 20]. Memory Th1 cells are important for providing long term protective immunity, and both Th17 and IL-17 play crucial role in both memory immunity inducing and making a balance between protection immunity and inflammation. A range of cytokines such as interleukin- (IL-) 12p40, IL-12p70, IL-23, IL-17, IL-12, INF-γ and antibodies are induce after animal vaccination with *B.melitensis* Rev 1 strain.

Brucella spp vaccines are live modified and induce both Th1, Th2 immune response, B and lymphocytes are involved. The development of protective immunity after brucellosis vaccination, is mostly correlated with Th1 immuneresponse and in particular with INF-γ production. A range of immunoglobulins, IgM, IgG1, IgG2, IgG3 are produced after vaccination, however humoral immunity do not correlate well with long-term protective immunity in vaccinated animals. Despite

that humoral immunity is not protective, it increase efficiencies of phagocytosis, activate complement and promote antibody-dependent cell mediated cytotoxicity by macrophages, neutrophils, natural killer cells, and neutralizing effect. CD4+ cells play a central role in coordinating and intensifying the adaptive immune response, while CD8+ lymphocytes kill infected cells [19]. Activation of Th1 cells could be demonstrate by measurement of INF-γ, however it is reported that its measurement is not satisfactory to predict protective immunity, better indicators for vaccine immunization are recommended level of IFN-γ, TNF-a, and IL-2 [3].

Detection of antibodies against O-Lipopolysacaride of *Brucella melitensis* is important for laboratory diagnosis and for immunoresponse monitoring after vaccination [4, 15]. Clinical diagnosis of Brucellosis is not reliable either in humans and or animals [15, 16]. Bacterial culturing remain "gold standard" methods, but it could be successfully applied during abortion, because the chance for positive culture on samples collected from positive animals is to low and it is not proper method for brucellosis disease surveillance [1, 4, 15, 16]. Molecular methods are expensive, require special sample and not available especially in developing country, such as Albania. At present diagnosis of brucellosis is based mostly on serological tests, either screening and confirmatory tests [1, 8, 12, 16, 18,].

Actual serological methods have some limitations, they are expensive, suffer from low specificity, are not sufficient to performed in different tissue samples, and are not able to discriminate infected from vaccinated animals [23]. Current *B. melitensis* Rev 1 vaccine is based on smooth strain and the alternative vaccine based on rough strain, *B. melitensis* B115 vaccine, is not available yet for using it in field [6, 22, 23].

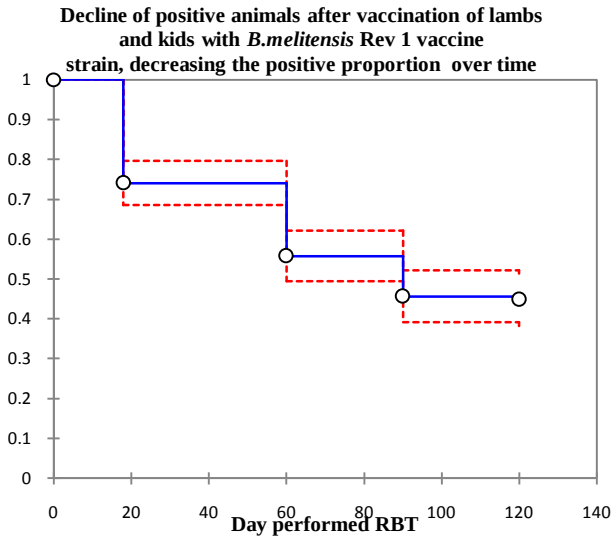

Figure 2 Kaplan Meier analyses for positive animals

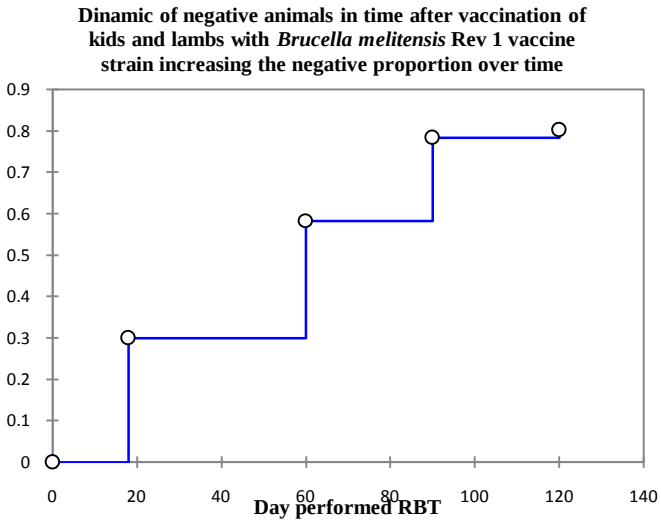

Figure 3 Kaplan Meier analyses for negative animals

The mass vaccination strategy applied in Albania, as in other countries, aimed to achieve a satisfactory protective immunity at level of small ruminant national herd [13]. In order to provide a generation immune small ruminant animals, vaccination of replacement animals must be strictly applied as is described in strategy. Despite the two years vaccination, and replacement vaccination will continue, brucellosis eradication take time to be achieved, so we guess that several infection foci will remain and sporadic outbreak may will occur. As for any disease, surveillance of humans and animals is essential for brucellosis control and its eradication. The serological surveillance is important for: epidemiological outbreak investigations, providing scientific data for designing proper control programmes, monitoring efficacy of vaccination strategy and evaluation of private veterinary operators (PVO) [15]. In our study the proportion of positive vaccinated replacement animal was very good and strong (Table 1). The post-vaccinal monitoring, at national level, was based on randomly selected from herds and flocks by national epidemiology unit. It is important to emphasize that proportion of positive replacement animals decreased soon after vaccination. After two months from vaccination proportion of RB positive animal was less

than 80%, as our results show. The value 80% represent a set value for accepted vaccination quality PVO work. If the vaccination data entry delayed in identification animal system, selection process could be postpone in time, and antibody titer could be too low so, laboratory results will negatively affect the performance of PVO. Persistence of antibody titers in vaccinated animals could be affected by age, dose, route of vaccination, and pregnancy status [12, 14, 15, 16, 18]. In general vaccination of replacement animal, ideally under six months of age, non- pregnant animal, and intraconjunctivly administration of vaccine aimed to make the vaccination safety, no risk for shedding bacteria in milk and decreasing the persistence of antibodies in sera of vaccinated animals.

For sheep and goat brucellosis, RBT and complement fixation test are suitable as screening and confirmatory diagnostic tests. RB is useful for early infected flocks, but its sensitivity is low especially in low-prevalence regions, however this could overpass by increasing the amount of sera [12, 15]. Both false positive and negative results could obtained by using RBT. The proportion of positive replacement animals vaccinated with *B.melitensis* Rev1, decrease soon after vaccination. There is a negative correlation coefficient (r =-0.982) between positive and negative animals in time. The Kaplan Meyer analyzes show this tendency for both lambs and kids (Figure 2 and Figure 3). Geometrical mean was calculated and it was 0.279, which could interpreted as, there is 27.9% average monthly decline of positive animals after vaccination with Rev 1

In conclusion, the vaccine *Brucella melitensis* Rev 1 strain used in small ruminant animals in Albania, induce a strong humoral response, however the proportion of positive vaccinated animals decrease progressively over time, and 4 months after vaccination all lambs were sero-negative; only one kid remain weakly (marked as one plus) sero positive in RB test. RB test could be used, with high confidence, for brucellosis surveillance four months after vaccination with *Brucella melitensis* Rev.1 strain vaccine administered intraconjunctively in animal between 3 and 7 months of age. In other hand, genetic methods have had provide: 1) scientific evidences for new classification of *Brucella* spp, 2) describing new species with public health importance, 3) Clarification of many gaps on pathogenesis, diagnostic methods, vaccination and vaccine programs. However, despite the remarkable achievements, there are several imperative problems to solve. Briefly, 1) It is a need to investigate, in large scale field trial, the duration of immunity in sheep and goats, 2) Producing a new generation vaccine (marker vaccine) which do not interfere with standard serological tests, 3) Improving current diagnostic methods for both sensitivity, specificity parameters, 4) improving the current control strategy of brucellosis for species host adopted and interspecies transmission, such as infection of *Brucella melitensis* infection in bovine species.

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