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# Research Article

## Detection of *Brucella Melitensis* Rev–1 Vaccinal Antibodies in Sheep in India

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ABSTRACT

In sheep, brucellosis is mainly caused by *Brucella melitensis* which is an important reproductive disease and is characterized by abortion in the fourth or fifth month of gestation, stillbirths and reproductive failure. The Rev.1 live *B. melitensis* vaccine is the most widely used vaccine in control programs against brucellosis in small ruminants in different parts of the world. This vaccine however shows a considerable degree of virulence and induces abortions. In India, *B. melitensis* Rev.1 vaccine for small ruminants is officially not recommended by the Government of India. Present study reports *B. melitensis* Rev.1 vaccinal antibodies detection in breeding sheep flock due to use of *Brucella melitensis* Revl vaccine. We investigated an organized sheep flock located in the southern part of India, consisting around 1200 sheep of breeds like Rambouillet and Bannur local breed ewes (600), Rambouillet lamb (300), crossbreds of Rambouillet and Dorper (200) and Rams (100) by random sampling of forty six sheep (vaccinated –20 and unvaccinated– 26) in order to detect antibodies against *B.melitensis* Rev–1 vaccinal strain. Among 20 vaccinated sheep serum samples tested, 19 (95%) and 13 (65%) and 19 (95%) were positive for anti *Brucella* antibodies by RBPT, SAT and iELISA respectively which is a major drawback of Rev–1 vaccine. This study further emphasized the need to initiate the control strategy in terms of suitable vaccines against *B. melitensis* in India in order to prevent import of Rev–1 vaccine by the farmers.



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### INTRODUCTION

Brucellosis is an important reproductive disease of sheep and goats characterized by abortion in the fourth or fifth month of gestation, stillbirths and reproductive failure. An estimated loss due to abortion and stillbirth in sheep and goats in India is Rs. 10,000 million/year (Gupta and Vihan, 2001). In sheep and goats, brucellosis is mainly caused by *Brucella melitensis*, a Gram–negative coccobacillus or short rod. This organism is a facultative intracellular pathogen. *B. melitensis* contains three biovars (biovars 1, 2 and 3). All three biovars cause disease in small ruminants, but their geographic distribution varies. *Brucella abortus* and *Brucella suis* infections also occur occasionally in small ruminants, but clinical disease seems to be rare. Control of brucellosis can be achieved by using vaccination to increase the population's resistance to the disease. Vaccination against *Brucella* infections in animals is usually performed by administration of the live attenuated smooth *Brucella* strains: *B.abortus* strain S19 and *B.melitensis* strain Rev.1. The non–smooth strain *B.abortus* RB51 has recently been introduced in some countries. *B.abortus* S19 and *B.melitensis* Rev.1 are proven effective vaccines against *B. abortus* in cattle and against *B.melitensis* and *B.ovis* in sheep and goats, respectively (Elberg, 1996; Nicoletti, 1990). Both vaccines have the disadvantages of causing abortion in a proportion of pregnant animals, and of being pathogenic for humans. However, their main disadvantage is the induction of O–PS specific antibodies that interfere with the widely used serological tests which employ S–LPS as antigen.

The Rev.1 live *B. melitensis* vaccine is the most widely used vaccine in control programs against brucellosis in small ruminants indifferent parts of the world. When properly used, the Rev.1 vaccine confers a long lasting protection against field infections in a high proportion of animals. This vaccine however shows a considerable degree of virulence and induces abortions when the first vaccine dose is administered during pregnancy. The antibody response to vaccination cannot be differentiated from the one observed after field infection, and this therefore impedes control programs. In India, *B. melitensis* Rev.1 vaccine for small ruminants is officially not recommended by the Government of India. However, *B. abortus* strain S19 for bovines is being used in few regions and it is recommended in the National Control Program on Brucellosis launched



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during 2011–12.The present study reports *B.melitensis* Rev.1 vaccinal antibodies detection in breeding sheep flock due to use of *B.melitensis* Rev.1 vaccine.

## MATERIALS AND METHODS

### *History*

An organized sheep flock located in the southern part of India, consisting of around 1200 sheep of breeds like Rambouillet ewes and Bannur local breed 300 each (600), Rambouillet lamb (300), crossbreds of Rambouillet and Dorper (200) and Rams (100). These sheep were maintained in semi–intensive system of rearing. Sheep were maintained in separate sheds as per age, sex, pregnancy status with good managemental and feeding practices and vaccinated against sheep pox, enterotoxaemia (ET), peste–des–petis of ruminants (PPR) and hemorrhagic septiceaemia (HS) annually. As reported, due to incidence of brucellosis, only non–pregnant breeding females of Rambouillet and bannur breed were vaccinated against brucellosis using *B. melitensis* Rev–1 vaccine (imported vaccine) at the age group of 4–12 months.

### *Samples*

Forty six (46) blood samples of 20 from vaccinated and 26 from unvaccinated 17 non–pregnant ewes and 9 rams in the age group of two to two and half years were collected. These animals received *B. melitensis* Rev–1 vaccine (procured by farmer from unknown sources) an year back at the age of 4–12 month. There were no incidences of late abortions in vaccinated flock whereas 3–5% abortions were regular feature in unvaccinated flock in the same farm. Five ml blood samples with and without anticoagulant were collected from 46 sheep along with deep vaginal swabs from 37 female animals in *Brucella* selective broth tubes (Pronadisa–Conda, Spain) containing antibiotic supplements.

### *Serological Studies*

Serum samples were subjected to rose bengal plate test (RBPT), serum agglutination test (SAT) and Indirect ELISA (iELISA). The SAT titre of ≥1:40 (80 IU/ml) was considered positive for brucellosis (Al Dahouk *et al*., 2003). *B.abortus* colored and plain antigens were obtained from the Institute of Animal Health and Veterinary Biological (IAH & VB), Bangalore, India. Indirect ELISA to detect antibrucella antibodies was carried out using smooth lipopolysaccharaide (sLPS) antigen as per the iELISA protocol described in OIE manual (OIE 2009) and standardized and being regularly used in our laboratory (Shome *et al*., 2007).

### *Isolation of Brucella Spp.*

Isolation of *Brucella* spp. was carried out using vaginal swabs from ewes (37) collected in *Brucella* selective broth tubes (Pronadisa–Conda, Spain) containing antibiotic supplements. Inoculated tubes were incubated with and without 10 per cent $CO_2$ at $37^{O}C$ for 72hrs. A loop full of broth culture from both the sets (broth) were streaked onto *Brucella* selective agar (Pronadisa–Conda, Spain) and incubated at 37 $^{0}C$ till the appearance of growth. The colonies were identified by the classical and molecular biotyping procedures (Alton *et al*., 1988).

### *Brucella Genus–Specific PCR*

For molecular characterization of *Brucella* spp amplification of *Brucella* genus–specific sequences were amplified by PCR using genus specific primers (Baily *et al*, 1992).The genomic DNA from 46 blood samples was extracted using DNAeasy blood and tissue kit (QiAgen, USA). The following primer pairs were used for the identification of genus *Brucella*: B4/B5 (B4 (F) TGGCTCGGTTGCCAATATCAA B5(R) CGCGCTTGCCTTTCAGGTCTG) for the expected amplified product of 223 bp (for the region of the sequence encoding a 31 kDa immunogenic bcsp31) as per Baily *et al.* (1992). The PCR reaction described briefly as: the reaction was carried out in 25µl reaction mixture of 12.5 µl 2x PCR–Master–Mix [0.05 units/µlTaq DNA polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mMdNTP (Fermentas)]. To make a final concentration of 1X, 1 µl of forward and reverse primers (12 pmol/µl), 10µl of DNA template, and nuclease free water was added to make 25µl final volumes. The DNA amplification reaction was performed in a Master Cycler Gradient Thermocycler (Eppendorf) with a preheated lid. The resultant PCR product was analysed by 1.5% agarose gel electrophoresis stained with ethidium bromide.

## RESULTS AND DISCUSSION

Brucellosis caused by *B. melitensis* is a significant problem in small ruminants; particularly in developing nations like India where small ruminant husbandry is gaining momentum due to market driven demand of meat and milk products, infections can be widespread. The relative importance of *B. melitensis* for sheep and goats varies with the geographic region, and can be influenced by husbandry practices and the susceptibility of sheep breeds in the region. Management practices and environmental conditions significantly influence the spread of infection. The administration of any live attenuated vaccine needs proper skill and technical knowledge in such management systems. The present study is an attempt to reveal the unauthorized use of *B.melitensis* Rev–1 strain as a vaccine in an organised farm.

The detection assays for goats and sheep are nearly the same as those for cattle because of the considerable genetic similarity between smooth strains of *Brucella* i.e. *B. Melitensis* and *B. abortus* (Nielsen, 2002).Among 20 vaccinated sheep serum samples tested, 19 (95%), 13 (65%) and 19 (95%) were positive for anti *Brucella* antibodies by RBPT, SAT and iELISA respectively. Similarly, in unvaccinated sheep sera samples, only 3 out of 26 (11.5%) positive by RBPT and SAT and 6 out of 26 (23%) by iELISA(Table 1). Out of three serological tests conducted, iELISA detected higher positives than the other two tests in both the groups. The higher positives detected in iELISA is due to the ability of the enzyme assay to detect very low levels of antibodies in the early or late stage of infection/ post vaccination while RBPT and SAT fails to detect the same (Guarino *et al*., 2000). Among 22 RBPT positive sera samples tested for SAT titres, significant SAT titres (› 1:40) in 16 (34.7%). In SAT too, specificity is reduced by nonspecific antibody thought to be IgM (OIE, 2008) and hence conventional screening tests are presently replaced by enzyme based assays which are sensitive and recommended for screening. RBPT is a screening test and is adequate for detecting infected herds or to guarantee the absence of infection in brucellosis free herds. Though it is used widely as screening test, the test has low specificity and hence RBPT positive sera has to be


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assessed further for SAT tires to interpret disease status (Smits and Kadri, 2005). Presently there is no objective criterion to decide whether cases exclusively detected or missed by either test represent false positive or negative reactions. This may account for the observed discrepancies in the cases of sheep which belong to a single farm of unknown infectious status. However, further confirmation of ELISA positive animals was much needed by some direct detection method like PCR. On screening of 46 blood samples by PCR, genus–specific 223bp product could not be amplified in both sero–positive and sero–negative samples and no isolations could be made from the 37 vaginal samples indicating only presence of antibody.

Table 1: Summary of results of immunoassays conducted in vaccinated and unvaccinated sheep

| Status of vaccination | No of animals | Sex | Vaccination age | Age at which blood collected | Results of immunoassays | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | RBPT | SAT titres >1:40 | ELISA | *Brucella* spp isolation | PCR |
| Vaccinated | 20 | F | 4–12 months | 14–24 months | 19 /20 (95%) | 13/20 (65%) | 19/20 (95%) | Nil | Nil |
| Unvaccinated | 26 | F=17 M=9 | NA | 10–32 months | 3 | 3 | 6/ 26 (30%) 3 each in male and female sheep | Nil | Nil |

The live attenuated *B melitensis* Rev.1 strain is presently recognized as the best available vaccine for the prophylaxis of brucellosis in sheep and goats. It has been now proved that vaccination of pregnant animals with a full dose of Rev.1 administered subcutaneously results abortion in many animals and produces long–lasting immune response.

The study clearly indicated the presence of vaccinal antibody in the vaccinated sheep suggesting the persistence of antibody beyond one year of vaccination. No late gestation abortions indicated the good protection in vaccinated ewes. In the unvaccinated 06 seropositive sheep (ewes: n=3, ram: n=3) cases were recorded by ELISA indicating either the exposure of these animals to *Brucella* infected material/ animals in the flock or introduction of new animals from brucellosis endemic flocks. The three breeding seropositive rams in the tested samples indicate greater chance of disease transmission within the farm.

The persistence of vaccinal antibody in *B.abortus* S19 vaccinated calves upto 180 days (Lord *et al*., 1998) and *B.melitensis* Rev.1 long–lasting antibody response has been reported (Blasco, 1997a). Like the RBPT, the ELISA is very sensitive, for detection of vaccine–induced antibody, and positive samples should be retested using a confirmatory and/or complementary test(s) like CFT. False–negative reactions may occur, usually due to prozone phenomenon, which may be overcome by diluting the serum or retesting after a given time (OIE 2008). The live attenuated *B.melitensis* Rev.1 strain given to replacement animals (3–5 months old) by the standard method ($1x10^9$cfu subcutaneously), the Rev.1 vaccine induces solid immunity against *B.melitensis*. However, infection in vaccinated animals by subcutaneous inoculation causes a generalised Rev.1low grade infection thus inducing an intense and long–lasting antibody response that interferes with subsequent serological screening (Elberg, 1996).Similar to *B abortus* infection in cattle, *B melitensis* can be transmitted from the dams to lambs or kids. A small proportion of lambs or kids can be infected *B melitensis*, but the majority of infections are probably acquired by consumption of colostrum or milk. These lambs or kids may have infections in the lymph nodes draining the gastro–intestinal tract and may shed *B melitensis* organisms in the faeces.

The preliminary sero–screening survey conducted during 2006–2010 on sheep samples received from seven states of India (n=1702), the prevalence of brucellosis was found to be 6.2% (106/1702) when tested by iELISA with the highest seroprevalence in the state of Karnataka and Rajasthan (data under publication). Because of increasing incidence of abortions in the sheep flocks and non–availability of the *B.melitensis* Rev.1 vaccine in the country, the farmer might have imported the vaccine from neighboring country to protect sheep against brucellosis.

*B. melitensis* widely accepted as the most virulent of *Brucella* spp., has proven to be a very difficult organism to eliminate and no country has been able to eradicate the disease following its widespread establishment. In general, mass immunization is indicated where the prevalence of infected animals is high. And it helps to rapidly establish a relatively immune stock, and reduces the level of abortions and excretors of *brucella*, thus reducing contamination of the environment and disease transmission (Kolar, 1995).Keeping the rise in both human and livestock brucellosis incidences ( Mantur and Amarnath ,2008) , both prophylaxis and complimentary measures needs to be adopted in India which has about 5.3% and 17% of world sheep and goat population, respectively (Livestock Census 2007).

*B. melitensis* Rev. 1 is currently the only approved vaccine available for protection against *B. melitensis* infection. Revl is pathogenic to humans via aerosol exposure or self–inoculation causing generalized brucellosis in affected individuals. Like all other *Brucella* vaccines, Revl can cause local hypersensitivity reactions in cases of accidental inoculation (Schurig *et al*., 2002).Erratic administration of vaccines or their use without adequate quality control is not effective and sometime poses threat to human population. Adequate protection is only possible if the vaccine quality is good and if the vaccines are administered to at least 80 % of the animals at risk (Garrido, 1992).The Rev.1 vaccine is a useful tool for the control of brucellosis in sheep and goats and to stop the infection of human beings. Its administration should be related to the epidemiological situation in order to be compatible with an eradication policy based on test–and–slaughter. The degree of


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attenuation of Rev.1 strain is not enough to allow its use without any restriction. Due to residual virulence it may induce abortions and also lead to persistent immune responses, which could interfere with classical methods of serological diagnostic tests. Even the Rev.1 mass vaccination strategy has two main draw backs:

I. The vaccination of pregnant animals with standard Rev.1 doses administered subcutaneously is followed by vaccine induced abortion in many animals (Alton and Elberg 1967; Elberg, 1981; Jiménez de Bagüéset al, 1989; Zundel et al, 1992; Blasco, 1997b). It has been stated that the capability of the Rev.1 strain to induce abortion is a phenomenon that depends on dose and on time of pregnancy when the females are vaccinated.

II. The vaccination of adult animals with standard Rev.1 doses administered subcutaneously induces a long–lasting serological response, making it difficult to discriminate the serological response evoked, when test–and slaughter eradication programs are simultaneously operated. The Rev.1 vaccine strain can cause infection in humans (Blasco and Díaz1993) and should therefore be handled and used with care.

*B. abortus* strain 19 and B. *melitensis* Rev.1 have been employed for several decades as the most potent vaccines available for cattle, and sheep and goats, respectively. These vaccines reduce abortion but not necessarily infection, and have been used primarily to lay the groundwork for eradication based on test and slaughter of infected animals. The intensive use of these vaccines in pilot experiments and in national eradication campaigns have revealed several adverse effects associated with their use. Moreover, field studies have recently shown the occurrence of horizontal transfer of the strain from vaccinated sheep to unvaccinated animals and its transformation into a rough form (World Health Organization, 1998). Finally the vaccine strains are fully virulent for humans and many accidental injection infections have been documented (World Health Organization, 1998). Present study indicated that if a farmer procuring and vaccinating without any biosafety measures and recommendation for vaccination in small ruminants may raise certain issues which needs to be addressed. The major issue is the lack of knowledge of unskilled persons regarding the *B. melitensis* Rev.1 vaccine strain hampers its standardization, leading to undesirable adverse effects when used in sheep and goat vaccination programs in future. This study further emphasized the need of rethinking on the part of policy makers to initiate the control strategy in terms of suitable vaccines against *B.melitensis* in India.

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