

Full Length Research Paper

## Identification of immunotolerance in the progeny of cows infected with *Brucella abortus*

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The progeny of cows infected with *Brucella abortus* could acquire the bacterium during their fetal stage and generate immune tolerance. In order to identify them, a clinical assay was conducted with two groups of seronegative calves. In Group I, seven bovine females received a standard S19 dose, and in Group II, eight males served as control. All animals were sampled seven times by official serological tests every 45 days. Also, complete blood with anticoagulant was collected and tested by the polymerase chain reaction (PCR) 45 and 135 days after vaccination. Results were analyzed by the test of proportions. In Group I, all the animals had their maximum antibodies title at the first sampling, but from the fourth sampling, their titles decreased until they became undetectable for the screening test. All animals in Group II remained negatives. Animals from both groups recorded negative with PCR. Significant differences ( $P < 0.05$ ) between groups were observed for seroconversion in the first two samplings. It was concluded that none of the tested animals were immunotolerant and that PCR may not be an appropriate method to demonstrate immunotolerance in some individuals.

**Key words:** Brucellosis vaccine, serological test, polymerase chain reaction (PCR), antibodies.

### INTRODUCTION

Bovine brucellosis is a zoonosis produced by *Brucella abortus* (World Health Organization, 2006). In Mexico the disease has been reported since 1900 (Foster et al., 2007; Moreno et al., 2002b). In dairy herds and cattle

brucellosis causes abortion, placental retention and metritis (Selem et al., 2010). The bacteria is shed or disseminated through fluids (Acha and Szyfres, 2003), even by apparently normal births and milk (López and Contreras, 2004; Memish and Balkhy, 2004). Calves and milk production losses have a negative impact on the economy and the public health (Mantur and Amarnath, 2008). In general, classical bovine brucellosis is caused by *Brucella abortus* smooth biovars. Smooth strains have

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a three regions lipopolysaccharide (LPS). Polysaccharide O is immunodominant and because of that, humoral immune response is primarily directed against it (Corbel, 1997; Robinson and Melling, 1993); however polysaccharide O is only useful for serological diagnosis (Díaz and Moriyón, 1989).

Most field strains and S19 vaccine correspond to smooth *B. abortus* biovar 1, both have polysaccharide O lateral chains antigens responsible for interfering with conventional serological diagnosis tests such as rose Bengal, rivanol, ring test and complement fixation (Bricker and Halling, 1995; Garcia-Yoldi, 2006). The only difference between field smooth strains and S19 vaccine of *B. abortus* is a 702 bp deletion at *Ery Locus* responsible from erythritol metabolism that is present in placental tissues in most of ruminants and facilitates gravid uterus colonization (Sangari et al., 1994). This genomic characteristic allows to differentiate field strains against S19 vaccine using a PCR procedure. Field *B. abortus* strains are very virulent (Crawford et al., 1988) and mannose molecules receptors in placental cells allow colonization during gestation (Moriyón and López-Goñi, 1998) with abortion as a consequence in infected cows. Also the bacteria could infect the fetus in the uterine life and about 10% of calves may be born infected but immunotolerant, thus resulting negatives to serological diagnosis tests and representing a persistent epidemiological risk because they may remain in the herd as negative ones (World Health Organization, 2006).

In infected animals, phagocytosis by monocytes protects the bacteria from antibodies and complement and allows it to reach lymph tissues and other lymphatic organs (Eze et al., 2000). In monocytes and neutrophils, respiratory burst and liberated oxygen free radicals (Canning et al., 1988) inhibit *Brucella* spp multiplication (Young et al., 1985); however in infected and immunotolerant animals those kinds of cells do not react and behave as an *in utero* infection. In such a case the infected progeny from positive cows must be eliminated (D'Pool et al., 2004). For the farmer, this means economical losses and the loss of certain desirable genetic traits. Thus the aim of this study was to vaccinate serum negative female calves born to seropositive cows in a persistent brucellosis infected herd, using a S19 *B. abortus* vaccine strain in order to monitor individual seroconversion ability, to demonstrate the presence of immunotolerant animals, and to discriminate between *B. abortus* infection by field strains and S19 vaccine strain presence.

## MATERIALS AND METHODS

### Study site

A clinical assay was conducted at a dual purpose cattle farm located in the municipality of Tlalixcoyan, in the central region of the state Veracruz, Mexico. This farm has been persistently infected over the years and has a 24% brucellosis seroprevalence as

determined by the official Mexican serological tests and confirmed by *B. abortus* biovar 1 isolation.

### Inclusion criteria

This study included 15 calves born to cows given as positive to brucellosis by official serological tests (SAGDR, 1996). Animals were divided into two groups; Group I was integrated by seven female calves and Group II by eight male calves. At the beginning of the experiment all animals were three to six month old. In order to avoid colostrum or infection interference after vaccination with *B. abortus* S19 strain all selected animals were tested by card test and remained as seronegative.

### Vaccination

All seronegative calves in Group I were vaccinated once with 1 to  $10 \times 10^{10}$  colony forming units (CFU) of the so-called "normal dose". A subcutaneous injection of *B. abortus* vaccine S19 strain was applied at the mid-neck section. Calves in Group II were not vaccinated and were left as control.

### Serological diagnosis and follow-up

The follow up was carried out at days 45, 90, 135, 180, 225, 270 and 315 post vaccination. For serological diagnosis blood samples were collected by venipuncture in vacuum tubes without anticoagulants and carried to the Microbiology Laboratory located at the Torreón del Molino Research Station of the Veterinary Medicine School, University of Veracruz, in Veracruz, Mexico, where serum was separated from all samples into Eppendorf™ type tubes and frozen at -20°C until processing by card and rivanol tests, used as screening and confirmatory, respectively (SAGDR, 1996).

### Molecular diagnosis

In order to obtain DNA from animals, blood samples were collected in vacuum tubes containing heparin as anticoagulant at days 45 and 135 post vaccination. Blood samples were carried out in a refrigerated container to the Microbiology Laboratory and remained frozen at -20°C until they were processed by the PCR procedure. The DNA extraction protocol used was that proposed by Leal-Klevezas et al. (1995, 2000) for a 25 µl reaction using a commercial kit (Go Taq™ Green Master Mix). This kit contains all the necessary elements for amplification. Oligonucleotides *Ery 1* (TTG GCG GCA AGT CCG TCG GT) and *Ery 2* (CCC AGA AGC GAG ACG AAA CG) were added. The amplification protocol to determinate the presence of S19 vaccine strain or field strains was that proposed by Sangari et al. (1994). The PCR obtained product was observed by electrophoresis in agarose gels (2% TAE) stained with 0.5 mg/mL EtBr in a UV transilluminator.

### Statistical analyses

Data were compared by the proportion test and 95% confidence intervals (CI) were calculated using Statistix software. Significant differences were declared at  $p < 0.05$ .

## RESULTS

During the studied period no animal in Group II

**Table 1.** Serological post vaccination follow-up using card (CT) and rivanol (RT) tests in female calves born to infected dams and vaccinated with *B. abortus* S19 strain vaccine.

ID.	45 day		90 day		135 day		180 day		225 day	270 day	315 day	
	CT	RT	CT	RT	CT	RT	CT	RT	CT	CT	CT	
686	+	1:50	+	1:25	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
692	+	1:25	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
679	+	1:100	+	1:25	+	1:25	NEG	NEG	NEG	NEG	NEG	NEG
681	+	1:400	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
693	+	1:50	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
694	+	1:50	+	1:25	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
695	+	1:400	+	1:400	+	1:200	+	1:100	NEG	NEG	NEG	NEG

\* NEG = negative; + = positive.

**Table 2.** Summary of seroconversion in follow-up samplings of calves born to infected dams and vaccinated or not with *B. abortus* S19 strain vaccine.

Sampling day	Positive	Negative
Group I at 45- day	7 <sup>a</sup>	0 <sup>a</sup>
Group I at 90- day	4 <sup>a</sup>	3 <sup>a</sup>
Group I at 135- day	2 <sup>b</sup>	5 <sup>b</sup>
Group I at 180-day	0 <sup>b</sup>	7 <sup>b</sup>
Group II in all sampling dates	0 <sup>b</sup>	8 <sup>b</sup>

<sup>a,b</sup> Different literals by row indicate significant difference at  $p < 0.05$ .

seroconvert, while all vaccinated in Group I did. As expected, highest antibodies titers occurred at day 45 post-vaccination as indicated by the diagnostic interpretation provided by Mariño-Jannaut (2000); however titers were not homogeneous in all animals. Two females gave a titer 1:400 using rivanol test as confirmatory test; one had 1:100, three 1:50, and one more 1:25 (Table 1). Antibody titers at posterior samplings progressively decreased becoming imperceptible by day 225 post-vaccination, so all female calves were considered as negatives. Seven samples were taken every 45-day for the two groups. However, only animals in the *B. abortus* vaccinated group showed antibodies titers decreasing over time. Animals in the non-vaccinated group were negative in all the sampling dates (Table 2). Results based on whole blood extracted DNA by PCR were discouraging. No desirable amplifiers were obtained from any sample in both groups and only amplicons in positive controls were observed as seen in Figure 1.

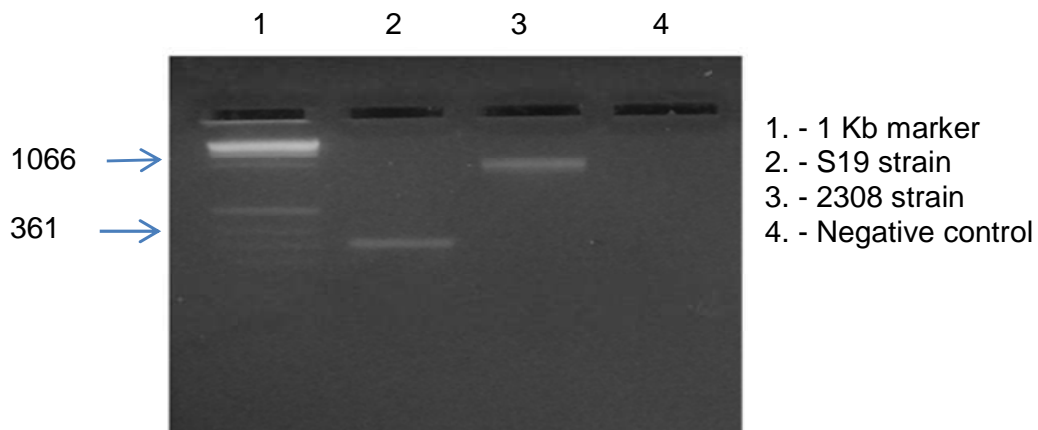
## DISCUSSION

Vaccination with S19 vaccine has an indisputable advantage to prevent infection and abortion (Peniche et al., 2009), but also the inconvenience of inducing seroconversion that interferes with conventional serological diagnosis. As a result, the use of this type of

vaccine shows a decreasing trend (Martínez et al., 2006); however, this apparent disadvantage could be useful. If a female from an infected herd is vaccinated with a S19 strain vaccine and then does not seroconvert, this can be considered as a clinical marker for immunotolerance against *B. abortus*, since a persistently infected animal may not be identifiable by the conventional official serological tests (Mariño-Jannaut, 2000).

Even though card test has 100% sensitivity (Dájer-Abimerhi et al., 1995), it cannot discriminate among infected and vaccinated animals. To offset this disadvantage females vaccinated with S19 strain vaccine must be inoculated between three to six month-old because younger animals retain post vaccination antibodies less time (Aparicio et al., 2003).

In this study vaccination with S19 vaccine stimulated antibodies against to lateral chains of the O polysaccharide; however antibodies titers were not homogeneous and at 90-day post vaccination titers decreased in some calves from Group I until they were negative to the screening test. For that reason it has been established that female calves vaccinated with *B. abortus* S19 strain around three month-old became seronegative two months after vaccination; on the contrary, animals vaccinated around six month-old take some six months to become seronegative (Mariño-Jannaut et al., 2000). This situation may explain the observed differences in titers reduction. Moreover all animals from both groups



**Figure 1.** Agarose gel electrophoresis (2% TAE) stained with 0.5 mg/ml EtBr and observed by an UV transilluminator.

remained with their dams from which elimination of *B. abortus* biovar field strains was previously demonstrated by milk bacteriological isolations.

In order to increase the chances of natural infection sampling intervals were adjusted to the maximum incubation period required for a calf to become sick, that is, 45 days (Robles et al., 2007). No animal in Group II seroconverted, hence the statistical analysis found statistical differences among samplings at 45 and 90 days post vaccination when compared to Group I ( $p < 0.05$ ) (Table 2). Samples for PCR were collected at 45 post vaccination day, when the highest antibodies titers are expected and the chances of bacterium circulation theoretically increase. However, it was not possible to identify the amplifier corresponding to the S19 vaccine strain in the vaccinated Group 1, or any field strain. So there is a possibility that infective field *B. abortus* strains had been sequestered by the immune system, and only could be evidenced when animals reach adulthood or, in the case of females, become pregnant (Plommet et al., 1973), because only at this time classical clinical signs like abortion could be observed.

For this reason, Wilesmith et al. (1978) has proposed that up to 5% of *B. abortus* cow reactors progeny could develop the illness when they become adults. Baek et al. (2005) used spleen macerates to identify intrauterine infection by *B. abortus* in mice progeny because whole blood PCR results were variable (Baek et al., 2005); however, Cevallos et al. (2008) have proposed to improve the brucellosis diagnostic procedure using whole blood assuming that primers election do not affect the test sensitivity, as suggested by Leal-Klevezas et al. (1995, 2000) and Sreevatsan et al. (2000) who have also used PCR with blood samples. In fact, Mosquera et al. (2008) corroborated the usefulness of PCR testing for blood brucellosis diagnosis with better results than using

milk samples. Biology molecular assays conducted to identify S19 vaccine strain using milk provided acceptable results. Martínez et al. (2006) identified shedding of the vaccine strain in the analyzed samples, representing a risk factor for public health.

PCR has been used with pure cultures to sequence (Crasta et al., 2008) and for diagnosis (Pavan et al., 2005), but information about its presence in blood is scarce. The presence of infected animals remaining in the population is the main factor favoring transmission and persistence of infection in a herd (Moreno et al., 2002a; Rentería et al., 2003); either not abortive cows reacting to brucellosis, or those who become infected during the gestation period (Baek et al., 2005). Efforts to eradicate animal brucellosis could be achieved by reducing or suppressing human incidence cases, which is also a responsibility for veterinarians (Nicoletti, 2002); so it is necessary to emphasize the disease importance and actions to control and eradicate. Even though latency period is rare, the progeny of infected cows are at risk of have it (Baek et al., 2005).

## Conclusion

The use of *B. abortus* S19 strain vaccine stimulated antibodies production in female calves born from seroreactors cows vaccinated against brucellosis. Vaccination in such calves caused diagnostic interference that remained at least until day 135 after vaccination day as measured by card and rivanol serological tests. However from day 185 on, no female calf vaccinated with normal dose showed serological evidence against antigens contained in the official serological tests, hence no female calf behaved as immunotolerant in the herd. Also, PCR was unable to

identify any DNA *B. abortus* carrier among the calves neither recognized DNA from S19 vaccine strain vaccinated female calves.

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## REFERENCES

- Acha PN, Szyfres B (2003). *Zoonoses and Communicable Diseases Common to Man and Animals*. Washington, D.C.; Third Edition. PAHO Scientific Publications, No 580. World Health Organization.
- Aparicio BA, Díaz AE, Hernández AL, Pérez GR, Alfonseca SE, Suarez GF (2003). Evaluación serológica y bacteriológica de un hato bovino con brucelosis y revacunado con dosis reducida de *Brucella abortus* cepa 19. *Téc Pecu Méx.* 41: 129-140.
- Baek KB, Lee OB, Hur J, Rahman MS, Lee IS, Kakoma I (2005). Evaluation of the Sprague-Dawley rat as a model for vertical transmission of *Brucella abortus*. *Can. J. Vet. Res.*, 69: 305-308.
- Bricker B.J. & Halling S.M. (1995). Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *J. Clin. Microbiol.*, 33: 1640-1642.
- Canning PC, Deyoe BL, Roth JA (1988). Oponin-dependent stimulation of bovine neutrophil oxidative metabolism by *Brucella abortus*. *Am. J. Vet. Res.*, 49:160-162.
- Cevallos FO, Motte E, Cedeño V, Carranza PM, Canchignia MHF, Saucedo ASG (2008). Implementación de la PCR para el Diagnóstico de la Brucelosis de Bovinos en el Ecuador. *Ciencia y Tecnología.* 1: 31-36.
- Corbel MJ (1997). Brucellosis: an overview. *Emerg. Infect. Dis.*, 3: 312-221.
- Crasta OR, Folkerts O, Fei Z, Mane SP, Evans C, Martino-Catt S, Bricker B, Yu G, Du L, Sobral B (2008). Genome sequence of *Brucella abortus* vaccine strain s19 compared to virulent strains yields candidate virulence genes. *PLoS ONE.* 3:e2193.
- Crawford RP, Adams GA, Williams JD (1988). Relationship of days in gestation at exposure and development of Brucellosis in strain 19 vaccinated heifers. *Am. J. Vet. Res.*, 49: 1037-1039.
- Dájer-Abimerhi A, Gutiérrez-Ruiz EJ, Zapata-Villalobos D, Honhold N, Villegas-Pérez SL (1995). Comparación de cinco pruebas serológicas para la detección de anticuerpos contra *Brucella abortus* y reporte preliminar del porcentaje de reactores positivos en hatos bovinos en Yucatán, México. *Rev Biom.* 6: 84-90.
- Díaz R, Moriyón I (1989). Brucellosis, clinical and laboratory aspects. In: Young EJ, Corbel MJ (eds.). *Laboratory techniques in the diagnosis of human brucellosis*. CRS, Boca Raton, Florida, pp. 73-83.
- D'Pool G, Rivera PS, Torres T, Pérez M, García A, Castejón O, Rojas N (2004) Prevalencia de brucelosis bovina mediante ELISA competitivo en el Municipio la Cañada de Urdaneta, estado Zulia, Venezuela. *Rev Cient, FCV-LUZ.* 14: 1-19.
- Eze MO, Yuan L, Crawford RM, Paranavitana CM, Hadfield TL, Bhatta C, Harjee AK, Warren RL, Hoover DL (2000). Effects of opsonization and gamma interferon on growth of *Brucella melitensis* 16M in mouse peritoneal macrophages *in vitro*. *Infect. Immun.*, 68: 257-263.
- Foster G, Osterman BO, Godfroid J, Jacques I, Cloeckeaert A (2007). *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int. J. Syst. Evol. Microbiol.*, 57: 2688-2693.
- García-Yoldi D, Marin CM, De Miguel PM, Munoz PM, Vizmanos JL, Lopez-Goni I (2006) Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clin. Chem.*, 52: 779-781.
- Leal-Klevezas D, Martínez-Vásquez I, López-Merino A, Martínez-Soriano J (1995). Single step PCR for the detection of *Brucella* spp. from blood and milk of infected animals. *J. Clin. Microbiol.*, 33: 3087-3090.
- Leal-Klevezas D, Martínez-Vásquez I, García-Cantu J, López-Merino A, Martínez-Soriano J (2000). Use of polymerase chain reaction to detect *Brucella abortus* biovar 1 in infected goats. *Vet Microbiol.*, 75: 91-97.
- López A, Contreras A (2004). *Brucella*. *Scand. J. Infect. Dis.*, 36: 636-638.
- Mantur GB, Amarnath SK (2008). Brucellosis in India - A review. *Bioscience.* 33:539-547.
- Mariño-Jannaut OC (2000). Brucellosis: metodologías diagnósticas e interpretación de resultados. *Rev MVZ-Cordoba.* 5: 57-60.
- Martínez CL, Verdugo RA, Hernández RC (2006). Identificación de la cepa vacunal *Brucella abortus* S19 en muestras de leche de vaca. *Vet. Mex.*, 37: 479-486.
- Memish AZ, Balkhy HH (2004). Brucellosis and International Travel. *J. Travel. Med.*, 11: 49-55.
- Moreno E, Cloeckeaert A, Moriyón I (2002a). *Brucella* evolution and taxonomy. *Vet. Microbiol.*, 90: 209-227.
- Moreno R, Rentería ETB; Searcy BR; Montaña GMF (2002b). Seroprevalencia y factores de riesgo asociados a brucelosis en hatos lecheros de Tijuana, Baja California. *Téc Pecu Méx.*, 40: 243-249.
- Moriyón I, López-Gofi I (1998). Structure and properties of the outer membranes of *Brucella abortus* and *Brucella melitensis*. *Int. Microbiol.*, 1: 19-26.
- Mosquera CX, Bernai VC, Muskus LC, Berdugo GJ (2008). Detección de *Brucella abortus* por PCR en muestras de sangre y leche de vacunos. *Rev. MVZ- Córdoba* 13: 1504-1513.
- Nicoletti P (2002). A short history of brucellosis. *Vet. Microbiol.*, 90: 5-9.
- OIE (2004). *Manual of diagnostic tests and vaccines for terrestrial animals*. Paris; World Organisation for Animal Health.
- Pavan ME, Nicola A, Grimoldi F, Cairo F (2005). Tipificación molecular de *Brucella abortus* cepa 19 y su aplicación al control de biológicos. *Rev. Argent Microbiol.*, 37: 122-125.
- Peniche A, Martínez D, Franco JL, Barradas F, Molina B, Gutiérrez EJ, Williams JJ, Morales F, Flores R (2009). Evaluation of vaccination with *Brucella abortus* S19 vaccine in cattle naturally infected with Brucellosis in productive systems found in the Mexican tropics. *Int. J. Dairy Sci.*, 4: 142-152.
- Plommet M, Fensterbank R, Renoux G, Gestin J, Philippon A (1973). Brucellose bovine experimentale XII. Persistencee a l'age adulte de l'infection congénitale de la genisse. *Ann. Rech Vét.*, 4: 419-435.
- Rentería ETB, Nielsen K, Licea NAF, Montaña GMF; Moreno R, JF (2003). Evaluación de un programa de control de la brucelosis bovina en hatos lecheros de Baja California. *Tec Pecu Méx.* 41: 275-282.
- Robinson A, Melling J (1993). Envelope structure and the development of new vaccines. *J. Appl. Bacteriol.*, 74: 43S-51S.
- Robles C, Cabrera R, Abalos P, Petray S (2007). Comparación de la respuesta inmune humoral y celular de las vacunas *Brucella abortus* cepa RB51 y cepa 19 en vaquillonas Hereford de Patagonia y su interferencia con el diagnóstico serológico de la enfermedad. *Rev. Med. Vet.*, (Buenos Aires). 88:102-106.
- SAGDR (1996). Norma Oficial Mexicana, NOM-041-ZOO-1995, Campaña Nacional contra la Brucelosis en los Animales. Secretaría de Agricultura, Ganadería y Desarrollo Rural. Diario Oficial de la Federación. 1 de julio de 1996. Mexico. Available at: <http://www.senasica.gob.mx/includes/asp/download.asp?idocumento=506&idurl=1258>.
- Sangari FJ, García-Lobo JM, Agüero J (1994). The *Brucella abortus* vaccine strain B19 carries a deletion in the erythritol catabolic genes. *FEMS Microbiol. Lett.*, 121: 337-342.
- Salem MN, Boyle SM, Srirangathan N (2010). Brucellosis: A re-emerging zoonosis. *Vet. Microbiol.*, 140: 392-398.
- Sreevatsan S, Bookout B, Ringpis F, Perumaalla V, Ficht A, Adams G, Hagius D, Elzer H, Bricker J, Kumar K, Rajasekhar M, Isloor S, Barathur R (2000). A multiplex approach to molecular detection of

- Brucella abortus* and/or *Mycobacterium bovis* Infection in Cattle. J. Clin. Microbiol., 38: 2602-2610.
- Wilesmith JW (1978). The persistence of *Brucella abortus* infection in calves: A retrospective study of heavily infected herds. Vet. Rec. 103: 149-153.
- World Health Organization (2006). *Brucellosis* in humans and animals. Washington DC. World Health Organization/Food and Agriculture Organization of the United Nations/ World Organization for Animal Health.
- Young EJ, Borchert M, Kretzertf L, Musher DM (1985). Phagocytosis and killing of *Brucella* by human polymorphonuclear leukocyte. J. Infect. Dis., 151: 682-688.