

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

186,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Recombinant Proteins as Antigens in Serological Diagnosis of Brucellosis

Magda Celina Navarro-Soto, Alberto Morales-Loredo, Genoveva Álvarez-Ojeda, Carlos Ramírez-Pfeiffer, Patricia Tamez-Guerra and Ricardo Gomez-Flores

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/61762>

Abstract

Brucellosis is considered the major zoonosis in developing countries. In susceptible animal species, diagnosis of brucellosis remains a challenge due to the variety of clinical signs that it shares with a wide range of diseases. At present, isolation of *Brucella* is considered the *gold standard* for diagnosis of brucellosis; because of its low sensitivity and becoming potentially hazardous to laboratory technicians, serology is used for the detection of specific antibodies induced by infection. However, since traditional methods commonly show drawbacks and do not differentiate between vaccinated and naturally infected animals, it is necessary to search and test immunoreactive molecules for specific diagnosis of *Brucella*-infected cattle, thus significantly reducing the killing of suspected herds mainly due to vaccination. Advances in biotechnology have allowed exploring the use of recombinant proteins as antigens to avoid the risk involved in the use of viable *Brucella* strains. The benefit of using recombinant proteins, such as outer membrane proteins (OMP) and other non-lipopolysaccharide (non-LPS) molecules as antigens, for serological diagnosis is promising, but there are still many concerns about their application. The aim of the present work is to show advances in the use of recombinant antigens and discuss their advantages and potential use as markers for the serological diagnosis in brucellosis.

Keywords: Serology, *Brucella*, diagnosis, recombinant protein, brucellosis

1. Introduction

1.1. Diagnosis of brucellosis

Brucellosis is a zoonosis caused by bacteria of the genus *Brucella*, which is characterized by gram-negative coccobacilli, intracellular facultative, and slow-growing bacteria that do not

have capsule or form spores [1]. Since clinical signs of brucellosis are not pathognomonic, diagnosis is dependent upon demonstration of the presence of *Brucella* spp. by microbiology, polymerase chain reaction (PCR), detection of its antigens, and demonstration of specific antibody or cell-mediated immune responses [2, 3]. At present, isolation of *Brucella* is considered the *gold standard*, but it has low sensitivity and is hazardous to laboratory technicians, therefore, serology is the most common method for the diagnosis of brucellosis.

Brucellosis serology is usually performed using antigens derived from *B. abortus* S19 strain, because the *Brucella* immunodominant antigens associated to the smooth-lipopolysaccharide (S-LPS) are to a large extent shared by all naturally occurring biovars of the so-called “smooth species,” *B. abortus*, *B. melitensis*, *B. suis*, *B. neotomae*, *B. ceti*, and *B. pinnipedialis* [4]. Unfortunately, because of the LPS sharing, conventional methods do not differentiate between the smooth *B. abortus* S19 vaccinated and naturally infected animals; in addition, there could be cross reaction with other gram negative bacteria, such as *Escherichia coli* O157:H7 and *Yersinia enterocolitica*, because of their LPS similarity [7], thus affecting the specificity (Sp) of the test. The rough RB 51 *B. abortus* vaccine lacks the OPS, which avoids false reactions.

Various tests are generally used to improve the final specificity, in which an initial screening assay, such as the buffered *Brucella* antigen, the rose Bengal (RBT), the buffered plate agglutination (BPAT) or the indirect ELISA tests, which possess high sensitivity and relative specificity, are used to select reactive samples, followed by a secondary confirmatory test, with higher specificity than the screening test, such as the complement fixation test (CFT), rivanol. Other modern tests can also be used including the fluorescence polarization assay (FPA) [3, 5, 8]. ELISA and FPA may be used for diagnosis [20], because of their high performance. On the other hand, since *B. canis* and *B. ovis*, known as “rough species” lack LPS, the CFT, agar gel immunodiffusion (AGID) test, and indirect ELISA (I-ELISA) using soluble surface antigens obtained from *B. ovis*, are preferred [6].

2. Immunodominant antigens

The following immunodominant antigens have been identified within the genus *Brucella*: (a) S-LPS (smooth lipopolysaccharide), (b) R-LPS (rough lipopolysaccharide), (c) outer membrane proteins (OMP), and (d) periplasmic and cytoplasmic proteins [9].

2.1. *Brucella* spp lipopolysaccharide

Lipopolysaccharide present in smooth species of *Brucella* comprises a glycolipid portion (lipid A) inserted in the outer membrane and a polysaccharide directed outward. The latter is divided into two sections: the core and the O-chain. *Brucella ovis* and *B. canis* naturally lack O-chain (OPS), whereas *B. melitensis*, *B. abortus*, and *B. suis* might lose it by mutation. S-LPS is markedly immunodominant on the serological response, therefore, most serological tests are focused on detecting antibodies to S-LPS and the use of bacterial suspensions or antigens without OPS leads to misdiagnosis [10].

2.1.1. *Brucella* outer membrane proteins

Although *Brucella* species are genetically closely related, there are differences in pathogenicity and host preference that may be favored, at least in part, by the outer membrane structure [11], mainly composed by LPS and OMPs. Since LPS of rough *Brucella* species (*B. ovis*, *B. canis*, and *B. abortus* RB51) lacks OPS, OMPs are more exposed on the surface and their role in the virulence of the bacteria has become very important in the search for antigens that can be used in the development of vaccines or diagnostic methods [12]. *Brucella* OMPs were initially identified and classified according to their molecular weight [13]. Thus, membrane proteins that are within group 1 have molecular weights between 88 and 94 kDa, group 2 (omp2a and omp2b) from 36 to 38 kDa, and group 3, comprising omp25 and omp31, from 25 to 27 and 31 to 34 kDa, respectively [14]. In *Brucella*, major OMPs are Omp25 and Omp31 (belonging to group 3), except in *B. abortus* where it has been demonstrated by molecular techniques the missing omp31 gene encoding this protein [15]. One study [16] reported that there is a good reactivity against Omp31 protein extracted from *B. ovis* in sheep sera, experimentally infected with the bacteria and with specific monoclonal anti-Omp31 antibodies, but little reactivity against Omp31 protein recombinant *B. melitensis*. This feature is attributed to the existence of differences in nine nucleotides between omp31 genes of both *Brucella* strains that strongly modify the antigenic properties of the encoded proteins [5], suggesting that this protein may be useful as antigen for the development of specific tests for the detection of infectious epididymitis caused by *B. ovis* in rams. Moreover, Omp28, also known as CP28 or BP26, has been identified as an immunodominant antigen in infected cattle, sheep, goats, and humans and could be useful for the detection of anti-*Brucella* humoral responses of infected animals [17].

On the other hand, 8 immuno-reactive non-LPS proteins were identified [18] using proteomics and then tested with *Brucella*-positive sera by ELISA and showed no cross-reaction to *Escherichia coli* O157: H7, *Yersinia enterocolitica*, or negative serum to *B. abortus*. Of these proteins, chaperonin GroES (21 kDa) and DnaK (71.2 kDa) showed high immune reactivity and therefore the greatest potential as diagnostic antigens. In addition, 18 immunodominant insoluble proteins of *Brucella abortus* were separated by two-dimensional electrophoresis (2-DE) and their immune-reactivity was tested against the antisera of cattle infected with *B. abortus*, or/and *Yersinia enterocolitica*, or the sera of non-infected cattle using Western blotting. A wide variety of these insoluble proteins were identified by MS/MS analysis as F0F1 ATP synthase subunit b, solute-binding family 5 protein, 28 kDa OMP, Leu/Ile/Val-binding family protein, histidinol dehydrogenase, hypothetical protein, twin-arginine translocation pathway signal sequence domain-containing protein, serine protease family protein, b-hydroxyacyl-(acyl-carrier-protein) dehydratase FabA, short chain dehydrogenase-/reductase carbonic anhydrase, ornithine carbamoyltransferase, leucyl aminopeptidase, cold shock DNA-binding domain-containing protein, Cu/Zn superoxide dismutase, and methionine aminopeptidase [19].

2.2. Recombinant antigens in serology of brucellosis

Advances in biotechnology have allowed exploring the use of recombinant proteins as antigens to avoid the risk involved in the use of viable *Brucella* strains. An extremely useful

application of proteomics to the diagnosis of infectious disease relies on the identification of novel diagnostic antigens by screening serum from infected and uninfected individuals against immunoblotted, 2-DE mapped proteomes of infectious agents [20]. Once those antigens are identified, due to easy production of recombinant proteins in prokaryotic systems, a wide variety of non-LPS molecules are cloned and expressed in the *Escherichia coli* system to obtain recombinant immune-reactive proteins. The most studied OMPs belong to group 3 (Omp25 and Omp31); recombinant Omp31 protein (rOmp31) obtained from *Brucella melitensis* expressed in the *Escherichia coli* system showed reactivity in *Brucella* positive sera, but not in *Brucella* negative sera, in a variety of animal species by iELISA. However, the sensitivity and specificity of each affected species showed significant difference [14]. iELISA performed with rOmp31 showed lower sensitivity (85%) and higher specificity (100%), compared with conventional rose Bengal plate test (RBPT), with 92% and 83%, respectively [21]. Other studies found that recombinant *B. melitensis* rOMP28 was immunoreactive to *Brucella* infected cattle, sheep, goat, and dog sera with a sensitivity of 88.7%, specificity of 93.8%, and accuracy of 92.9% by iELISA, demonstrating that it could be used as an antigen for diagnosis of brucellosis in domestic animals [22].

Furthermore, *B. abortus* Omp28 coding gene was cloned and expressed using the pMAL system, and rOmp28 was evaluated for its potential use in the serodiagnosis of bovine brucellosis by iELISA and the latex bead agglutination test (LAT). The sensitivity, specificity, and accuracy were 96.7%, 95.4%, 96.2% in iELISA and 77%, 80.6%, and 78.5% in latex bead agglutination test, respectively [23]. In addition, recombinant BP26 was produced in the *E. coli* system and tested by iELISA, but it resulted less useful than iELISA using the *B. ovis* hot saline (HS) extract as antigen [24, 25]. On the other hand, ribosome recycling factor protein CP24 and *Brucella* lumazine synthase (BLS) showed antigen-antibody interaction by iELISA, using brucellosis positive sera, and therefore it could be considered as a potential alternative diagnostic [26].

Other studies focused in the type IV secretion system (T4SS) encoded by the *virB* locus, located on chromosome II, including *virB1* to *virB12* [27], obtained recombinant VirB5 protein by a prokaryotic expression system, which was used to detect anti-*Brucella* antibodies by ELISA, in both standard brucellosis-positive serum and cattle sera samples; the results showed that recombinant VirB5 protein had good immune-reactivity [27]. In addition, in order to investigate the practical value of VirB5 in clinical applications, serum samples from cattle were screened using the VirB5-ELISA; the sensitivity of the VirB5-ELISA was 88.2% and the specificity was 97.8%. In all test samples, the accuracy reached 94.8%. Thus, these results confirmed the importance of VirB5 as a suitable antigen and VirB5-ELISA as screening test for the serological diagnosis of bovine brucellosis [27]. Another study was developed by Rolan et al. in 2008 [28], evaluating recombinant VirB1, VirB5, VirB11, and VirB12 by antibodies in sera from experimentally infected mice and goats by iELISA. Antibody responses to VirB12 but not to VirB1, VirB5, or VirB11 were detected in mice experimentally inoculated with *B. abortus* and goats experimentally infected with *Brucella melitensis*.

3. Conclusion

At present, FAO-OIE-WHO work together in strategies to prevent a worldwide emergent, re-emergent, and cross-border spread of human and animal infectious diseases [29]. Brucellosis remains a major zoonotic disease in the world and its control and eradication will be possible only with the complete collaboration of all sectors involved in health and animal production. As a significant part of the strategy, *One World-One Health* (OWOH) [30] involves early diagnosis of infected animals. Conventional serological tests have performance differences due to a variety of factors, including sample condition, vaccinated status, wide spread, and others. Therefore, it is necessary to search immune-reactive molecules that prevent faulty results that could compromise campaigns of control and eradication of this disease. The aim of this work is to bring together advances in the use of recombinant antigens, their problems, and perspectives as potential markers for the serological diagnosis in brucellosis. It is known that a test based on recombinant proteins would allow better standardization of the assay, compared with more complex whole-cell antigen preparations currently in use, and hence overcome the limitations associated with the use of LPS-based antigens, but finding results still not optimal (Table 1). Many of the failures in performance found in the analyzed studies might originate due to denaturing conditions in purification or Western blotting that could affect on the tertiary structure of the recombinant protein, and hence to immune-dominant epitopes, or by a low adherence to polystyrene plaque in the ELISA test. In addition, the expression of immune-dominant proteins could be different between *in vitro* and *in vivo* culture conditions and then results obtained would be distinct in sera from animals naturally infected with field strains compared with experimentally infected animals. Therefore, the search of alternative purification techniques to the ones currently used that retains the structural integrity of the protein is essential. Furthermore, standardization of homogeneous diagnostic tests as FPA [31], which minimize the subjective factor involved in the interpretation of results found in agglutination tests, could be helpful in the control and eradication programs worldwide.

Test	Sensitivity (%)	Specificity (%)	Animal specie
RBT ^a	21.0–98.3	68.8–100	Cattle
iELISA ^a	92.5–100	90.6–100	Cattle
CFT ^a	23.0–97.1	30.6–100	Cattle
FPA ^a	99.0–99.3	96.9–100	Cattle
FPA ^b	85.7%	99%	Goat
rOmp31-iELISA ^c	85	100	Goat
<i>B. melitensis</i> rOMP28-iELISA ^d	88.7	93.8	Cattle, sheep, goat, and dog
<i>B. abortus</i> rOMP28-iELISA ^e	96.7	95.4	Cattle
rVirB5-ELISA ^f	88.2	97.8	Cattle

a. [8]; b; [31]c. [21]; d. [22]; e. [23]; f. [27].

Table 1. Sensitivity and specificity of serological tests for brucellosis with conventional and recombinant antigens

Author details

Magda Celina Navarro-Soto¹, Alberto Morales-Loredo², Genoveva Álvarez-Ojeda⁴, Carlos Ramírez-Pfeiffer³, Patricia Tamez-Guerra¹ and Ricardo Gomez-Flores^{1*}

*Address all correspondence to: rgomez60@hotmail.com

1 Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas, Laboratorio de Inmunología y Virología, Unidad de Inmunobiología y Acarreadores de Drogas, San Nicolás de los Garza, Nuevo León, México

2 Universidad Autónoma de Nuevo León, Centro de Desarrollo de Agronegocios, Escobedo, Nuevo León, México

3 Universidad México Americana del Norte, Coordinación de Investigación Institucional, Reynosa, Tamaulipas, México

4 Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. Campo Experimental Río Bravo, Tamaulipas, México

References

- [1] Cardoso PG, Macedo GC, Azevedo V, Oliveira SC. Brucella spp noncanonical LPS: structure, biosynthesis, and interaction with host immune system. *Microb Cell Fact* [Internet]. 2006 Jan [cited 2011 Jul 22];5:13. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1435926&tool=pmcentrez&rendertype=abstract>
- [2] Gopaul KK, Koylass MS, Smith CJ, Whatmore AM. Rapid identification of Brucella isolates to the species level by real time PCR based single nucleotide polymorphism (SNP) analysis. *BMC Microbiol* [Internet]. 2008 Jan [cited 2015 Mar 21];8(1):86. Available from: <http://www.biomedcentral.com/1471-2180/8/86>
- [3] OIE. Bovine brucellosis. *Terr Manual Bov Brucell* [Internet]. 2012;1:616–50. Available from: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.03_BOVINE_BRUCELL.pdf
- [4] Cloeckert A, Vizcaino N, Paquet JY, Bowden RA, Elzer PH. Major outer membrane proteins of Brucella spp: past, present and future. *Vet Microbiol* [Internet]. 2002;90:229–47. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12414146
- [5] Garin-Bastuji B, Blasco JM, Marín C, Albert D. The diagnosis of brucellosis in sheep and goats, old and new tools. *Small Rumin Res* [Internet]. 2006 Mar [cited 2015 Mar

- 21];62(1-2):63–70. Available from: <http://www.sciencedirect.com/science/article/pii/S0921448805003378>
- [6] OIE. Ovine Epididymitis [Internet]. Terrestrial Manual 2009. Ovine Epididymitis (*Brucella ovis*). 2009 [cited 2015 Jan 1]. p. 1–9. Available from: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.07.09_OVINE_EPID.pdf
- [7] Caroff M, Bundle DR, Perry MB, Cherwonogrodzky JW, Duncan JR. Antigenic S-type lipopolysaccharide of *Brucella abortus* 1119-3. *Infect Immun* [Internet]. 1984 Nov 1 [cited 2015 Mar 21];46(2):384–8. Available from: <http://iai.asm.org/cgi/content/long/46/2/384>
- [8] Nielsen K. Diagnosis of brucellosis by serology. *Vet Microbiol*. 2002. p. 447–59.
- [9] Mejía P, Díaz E, Salas E. Identificación de las proteínas de 35 y 38 kDa específicas de *Brucella ovis* Identification of *Brucella ovis*-specific proteins of 35 and 38 kDa. *Téc Pecu Méx* [Internet]. 2004;42(2):277–85. Available from: <http://www.tecnicapecuaria.org.mx/trabajos/200405285107.pdf>
- [10] Guzman-Verri C, Manterola L, Sola-Landa A, Parra A, Cloeckaert A, Garin J, et al. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the Rhizobiaceae. *Proc Natl Acad Sci* [Internet]. 2002 Sep 6 [cited 2015 Mar 21];99(19):12375–80. Available from: <http://www.pnas.org/cgi/content/long/99/19/12375>
- [11] Martín-Martín AI, Caro-Hernández P, Sancho P, Tejedor C, Cloeckaert A, Fernández-Lago L, et al. Analysis of the occurrence and distribution of the Omp25/Omp31 family of surface proteins in the six classical *Brucella* species. *Vet Microbiol* [Internet]. 2009 May 28 [cited 2012 Jul 19];137(1-2):74–82. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19135812>
- [12] Caro-Hernández P, Fernández-Lago L, de Miguel M-J, Martín-Martín AI, Cloeckaert A, Grilló M-J, et al. Role of the Omp25/Omp31 family in outer membrane properties and virulence of *Brucella ovis*. *Infect Immun* [Internet]. 2007 Aug [cited 2012 Jul 19];75(8):4050–61. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1952020&tool=pmcentrez&rendertype=abstract>
- [13] Moreno E, Moriyó I. *The Prokaryotes* [Internet]. Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. Springer New York; 2006 [cited 2012 Nov 1]. 315-456 p. Available from: <http://www.springerlink.com/index/10.1007/0-387-30745-1>
- [14] Cassataro J, Cassataro J, Velikovskiy CA, Velikovskiy CA, Barrera S De, Barrera S De, et al. A DNA Vaccine Coding for the. *Microbiology*. 2005;73(10):6537–46.
- [15] Cloeckaert A, Verger J-M, Grayon M, Vizcaíno N. Molecular and immunological characterization of the major outer membrane proteins of *Brucella*. *FEMS Microbiol Lett* [Internet]. 1996;145(1):1–8. Available from: <http://www.sciencedirect.com/science/article/pii/0378109796003734>

- [16] Kittelberger R, Cloeckart A, Ferna L, Mari CM. Minor nucleotide substitutions in the omp31 gene of *Brucella ovis* result in antigenic differences in the major outer membrane protein that it encodes compared to those of the other *Brucella* species. 2001;69(11):7020–8.
- [17] Gupta VK, Kumari R, Vohra J, Singh SV, Vihan VS. Comparative evaluation of recombinant BP26 protein for serological diagnosis of *Brucella melitensis* infection in goats. *Small Rumin Res*. 2010;93:119–25.
- [18] Kim J-Y, Sung S-R, Lee K, Lee H-K, Kang S-I, Lee JJ, et al. Immunoproteomics of *Brucella abortus* RB51 as candidate antigens in serological diagnosis of brucellosis. *Vet Immunol Immunopathol* [Internet]. 2014 Aug 15 [cited 2015 Mar 22];160(3-4):218–24. Available from: <http://www.sciencedirect.com/science/article/pii/S0165242714001226>
- [19] Ko KY, Kim JW, Her M, Kang S Il, Jung SC, Cho DH, et al. Immunogenic proteins of *Brucella abortus* to minimize cross reactions in brucellosis diagnosis. *Vet Microbiol*. 2012;156:374–80.
- [20] OIE. Biotechnology in the Diagnosis of Infectious Diseases and Vaccine Development. *Terr Man* [Internet]. 2009;(138):1–25. Available from: http://web.oie.int/eng/normes/mmanual/2008/pdf/1.1.07_BIOTECHNOLOGY.pdf
- [21] Gupta VK, Verma DK, Singh SV, Vihan VS. Serological diagnostic potential of recombinant outer membrane protein (Omp31) from *Brucella melitensis* in goat and sheep brucellosis. *Small Rumin Res* [Internet]. 2007 Jul [cited 2015 Mar 21];70(2-3):260–6. Available from: <http://www.sciencedirect.com/science/article/pii/S0921448806000265>
- [22] Chaudhuri P, Prasad R, Kumar V, Gangaplara A. Recombinant OMP28 antigen-based indirect ELISA for serodiagnosis of bovine brucellosis. *Mol Cell Probes* [Internet]. 2010 Jun [cited 2015 Mar 21];24(3):142–5. Available from: <http://www.sciencedirect.com/science/article/pii/S0890850809000851>
- [23] Lim JJ, Kim DH, Lee JJ, Kim DG, Min W, Lee HJ, et al. Evaluation of recombinant 28 kDa outer membrane protein of *Brucella abortus* for the clinical diagnosis of Bovine Brucellosis in Korea. *J Vet Med Sci*. 2012;74(6):687–91.
- [24] Seco-Mediavilla P, Verger J-M, Grayon M, Cloeckart A, Marín CM, Zygmunt MS, et al. Epitope mapping of the *Brucella melitensis* BP26 immunogenic protein: usefulness for diagnosis of sheep brucellosis. *Clin Diagn Lab Immunol*. 2003;10:647–51.
- [25] Zygmunt MS, Baucheron S, Vizcaino N, Bowden RA, Cloeckart A. Single-step purification and evaluation of recombinant BP26 protein for serological diagnosis of *Brucella ovis* infection in rams. *Vet Microbiol*. 2002;87:213–20.
- [26] Thepsuriyanont P, Intarapuk A, Chanket P, Tunyong W, Kalambaheti T. ELISA for brucellosis detection based on three brucella recombinant proteins. *Southeast Asian J Trop Med Public Health*. 2014;45:130-141.

- [27] Tan W, Wang X, Nie Y, Wang C, Cheng L, Wang X, et al. Recombinant VirB5 protein as a potential serological marker for the diagnosis of bovine brucellosis. *Mol Cell Probes* [Internet]. 2012 Jun [cited 2015 Mar 21];26(3):127–31. Available from: <http://www.sciencedirect.com/science/article/pii/S0890850812000357>
- [28] Rolán HG, Den Hartigh AB, Kahl-McDonagh M, Ficht T, Adams LG, Tsolis RM. VirB12 is a serological marker of *Brucella* infection in experimental and natural hosts. *Clin Vaccine Immunol*. 2008;15:208–14.
- [29] FAO/OIE/WHO. The FAO-OIE-WHO Collaboration. A Tripartite Concept [Internet]. 2010;(April):1–8. Available from: http://www.who.int/influenza/resources/documents/tripartite_concept_note_hanoi_042011_en.pdf
- [30] Hinchliffe S. More than one world, more than one health: Re-configuring interspecies health. *Soc Sci Med* [Internet]. 2015 Mar [cited 2015 Mar 22];129:28–35. Available from: <http://www.sciencedirect.com/science/article/pii/S0277953614004365>.
- [31] Ramírez-Pfeiffer C, Díaz-Aparicio E, Gomez-Flores R, Rodríguez-Padilla C, Morales-Loredo A, Álvarez-Ojeda G. Use of the *Brucella melitensis* native hapten to diagnose brucellosis in goats by a rapid, simple, and specific fluorescence polarization assay. *Clin Vaccine Immunol*. 2008;15:911-915.

