

## Management of an Outbreak of Brucellosis due to *B. melitensis* in Dairy Cattle

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Bovine brucellosis is an endemic infectious disease which can affect cattle productivity and welfare negatively, as well as human health. Sufficient knowledge on its epidemiology, particularly on species and biotypes of *Brucella* at regional scale are important to set up and implement efficient control measures against brucellosis. An outbreak of cattle brucellosis in a dairy cattle in Xinjiang of China. A total of 295 cattle examined, 11 were positive and 284 negative in serological tests of brucellosis. *Brucella* strains were isolated from seropositive milk samples in the farm. Species and biovars of the isolated *Brucella* strains from outbreaks were determined based on molecular (virB8-PCR and AMOS-PCR) and bacteriological methods. The examinations revealed that *B. melitensis* biovar 3 as the aetiological agent of brucellosis in the farm. The study confirmed that test-and-slaughter strategies were applied to control the outbreak in 3 years depending on the low infection level. Moreover, the results indicated that combination of molecular tools could help to identify infection and evaluate effective measures for disease reduction in order to determine preventive strategies.

**Key words:** Cattle; Brucellosis; *B. melitensis*; PCR; Control.

Brucellosis is one of the most important zoonotic diseases worldwide, and is of particular significance in developing countries. The disease, which results in significant economic losses and serious health problems, is caused by Gram negative coccobacilli bacteria of the genus *Brucella*. (Donev 2010). Brucellosis remains of particular concern due to the transmission of this severe disease to humans. The infection can result from ingestion of contaminated dairy products, inhalation, and via open skin wounds or mucous membranes from a shedding animal or infected environment (Karyn *et al.*, 2012).

*Brucella* spp. are divided into six classical *Brucella* species by their strong affiliation to specific natural hosts: *B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *Neotomae*. With the exception of *B. ovis* and *B. neotomae*, all other species are capable of infecting man (Osterman and Moriyon 2006). *B. melitensis* is the most frequently isolated species from all domestic ruminants (Julio *et al.* 2011; Curro *et al.* 2012). And it is the most pathogenic and invasive species for human brucellosis (Seleem *et al.* 2010). Clinical symptoms of brucellosis in humans include undulant fevery arthralgia, myalgia, abdominal pain, headaches and personality changes (Cutler *et al.* 2005; Valderas and Roop 2006). Therefore, as human brucellosis is essentially a zoonotic disease, control and prevention of brucellosis in animals is essential for eradicating the disease in man.

Brucellosis has been systematically surveyed in China since it was confirmed in the 1950s. Analysis of the *Brucella* strains isolated

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from human and animals showed that the most common species was *B. melitensis*, followed by *B. abortus* and *B. suis*. Only a small proportion of the cases are caused by *B. ovis* and a typical *Brucella* strains (Zhong *et al.*, 2013). From 2007-2011, *B. melitensis* was the most prominent species associated with outbreaks (Wang *et al.* 2007; Li *et al.* 2011). For the regions where at least two species exist in Xinjiang, the predominant strains were *B. melitensis* with either *B. abortus* or *B. suis*.

The control of brucellosis requires a major and prolonged effort and confirmation of the spread of the infection is essential when planning a control program. Therefore, in the study, species and biovars of the *Brucella* strains from outbreaks were determined based on molecular (virB8-PCR and AMOS-PCR) and bacteriological methods. According to the result, test and slaughter policy were applied to control the outbreak of brucellosis in the farm for 3 years. All results indicated that it was a key point to determine effective strategies for performance the brucellosis eradication program according to disease epidemiology on a region.

## MATERIALS AND METHODS

### *Brucella* strains and selective medium

*B. abortus* 544A strain and *B. melitensis* 16M strain were provided by Laboratory (Veterinary Research Institute, Academy of Animal Science, Urumuqi, China). The *Brucella* selective medium was manufactured using a *Brucella* medium base ((BBL™ *Brucella* Agar, BD Biosciences, USA) and a *Brucella* selective supplement (Oxoid, SR0209E). All *Brucella* work was conducted in a biosafety level 3 laboratory.

### Samples

The Farm was a large dairy (about 295 animals) that shared milk transport in a town. It located in an area of Xinjiang in China, and there were no brucellosis vaccination had been performed on this area. On March 2011, brucellosis was confirmed in a man that worked in the farm. Almost at the same time, routine tests performed as part of the routine monitoring of brucellosis program. Serum samples were collected from each cow in the farm and used for serum agglutination test (SAT) and indirect ELISA (Haerbing pinghe Co. China) of brucellosis. The positive of

serological analysis revealed 11 reactors at SAT and iELISA detection of the outbreak. The raw milk samples of 8 were collected from brucellosis-positive cows based on SAT and milk ring test (MRT), while the raw milk samples were stored at -20 °C in a refrigerator for culture.

### Bacterial culture and isolation

Bacteria were isolated from raw milk of the cow with serological-positive. 300 µL of the raw milk samples were cultured on *Brucella* selective agar plates which contained *Brucella* selective supplement. Each raw milk sample were cultured on 2 plates. Then the plates were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere and growth were evaluated after 4 days. The suspected *Brucella* colonies were picked into *Brucella* broth (TSB), which incubated at 37 °C under 5% CO<sub>2</sub> atmosphere of for 2 days. The cultures were inactivated at 100°C for 30 min and used to as a PCR template.

### Identification of *Brucella* by PCR

Primer designs with primer 5.0 molecular software. The primers of the detection of *Brucella* genus by VirB8-PCR and differentiation of the species of *B. abortus* and *B. melitensis* by AMOS-PCR were selected as follows:

VirB8-F: 5'-ATGTTTGGACGCAAACAATCTC-3', VirB8-R: 5'-TCATTGCACCACTCCCATTTCT-3' AMOS-A: 5'-GACGAACGGAATTTTCCAATCCC-3', AMOS-B: 5'-AAATCGCGTCCTTGCTGGTCTGA-3', IS711: 5'-TGCCGATCACTTAAGGGCCTTCAT-3'

The PCR process was performed in the reference (Yi *et al.*, 2013).

### Biochemical test

The culture of the *Brucella* strain involved in the outbreak was identified with the *Brucella* biotyping according to biochemical test. Subsequent the tests for hydrogen sulfide production, carbon dioxide requirement, growth on media containing basic fuchsin and thionin (20 µg/ml), and agglutination by monospecific antisera (A, M, R; Beijing) were carried out. The work was performed in Chinese Center for Disease Control and Prevention.

### Farms management system

In the farm, test-and-slaughter strategies were applied to control the outbreak when 11 reactors of brucellosis were detected and the *Brucella* strain caused the outbreak was determined. Positive animals of 11 cattle were

slaughtered. Serological analysis were repeated on day 30 post-detection of the outbreak, revealing 2 reactors at SAT and iELISA. The 2 cattle were also slaughtered and buried for avoiding the spread of the disease. And then the farm were carried out strict disinfection, improving management practices and movement control for disease control and prevention. There were no cattle introduced to the farm since the outbreak. Periodical testing of all animals and slaughter strategies were applied to control the outbreak in the farm in 3 years.

### RESULTS AND DISCUSSION

A history of brucellosis infection in a herd is also often considered a risk factor in epidemiological surveys. Serological data may be misleading due to cross-reactivity between *Brucella* and other Gram-negative bacteria. Further

more, serological methods cannot differentiate between vaccinated and infected animals. Thus, the isolation of bacteria is considered as a gold standard for the diagnosis of brucellosis, which the *Brucella* were isolated from aborted fetuses, hygroma fluids, milk, and vaginal swabs. *Brucella* from milk samples may be improved if more than one culture medium is used (Moon et al. 2010). In this study, selective media were used and a high rate of isolation from milk is achieved. The result showed that the medium with *Brucella* supplement was suitable for isolating *Brucella* and improving the performance of the bacteriological diagnosis of brucellosis. Therefore, it can offer epidemiologic traceback sources for the dissemination or transfer of diseases and applied as a useful tool for important control measures in disease eradication programs.

PCR-based methods for detecting *Brucella* genomic DNA was used widely for its biosafety (Bricker, 2002). Bacterial type IV secretion systems (T4SS) are essential virulence factors for *Brucella*, which T4SS assembly and virulence requires dimerization of VirB8 (Paschos et al 2006). Therefore, It was used to be a diagnosis method of the genus *Brucella*. 10 suspected *Brucella* colonies from 5 raw milk samples were identified by VirB8-PCR (Zhong., et al, 2008). The standard strains of *B. abortus* 544A and *B. melitensis* 16M as the positive control, the result showed that the 8 isolations from 4 samples were amplified fragment of approximately 719 bp specificity (Fig.2). The result proved that the suspected isolations were *Brucella* genus by PCR.

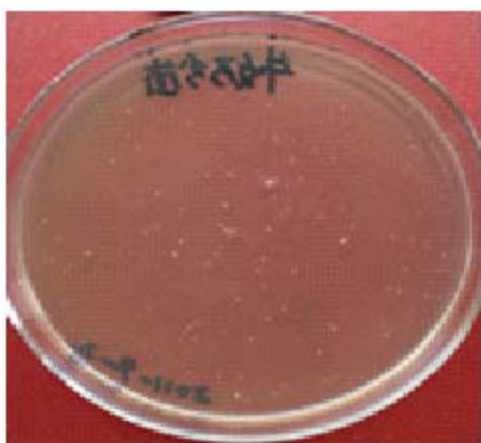
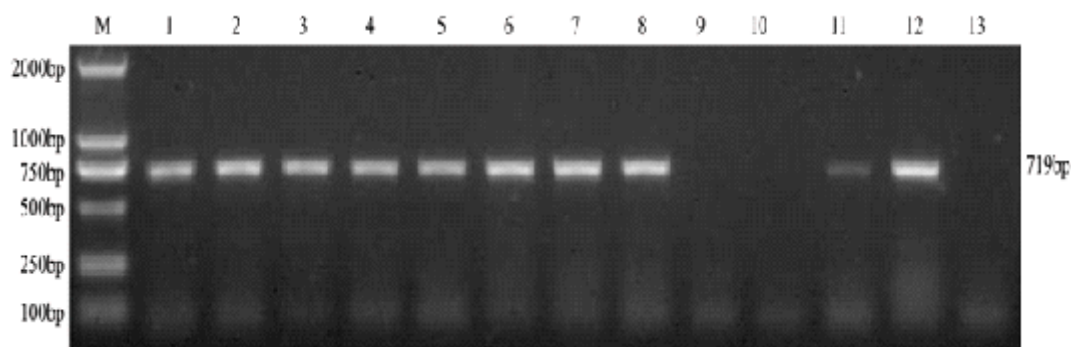


Fig. 1. Morphology of bacteria culture from raw milk sample

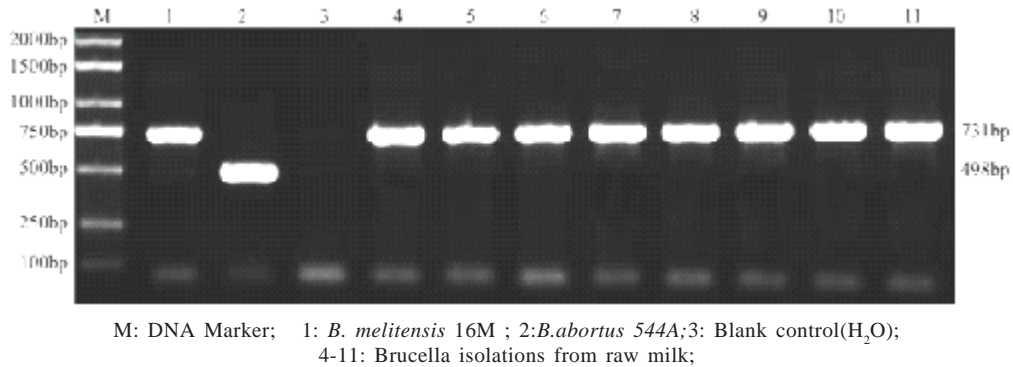


M: DNA Marker; 1-10: Suspected isolations in raw milk; 11: *B. abortus* 544A; 12: *B. melitensis* 16M ;13: Blank control(H<sub>2</sub>O) ;

Fig. 2. Identification of Brucella in raw milk by VirB8-PCR

Molecular methods like PCR assay enable safe identification of the bacteria at species level (Zhong *et al.*, 2011). In history, researches were proved that the prevalence of *brucella* strains were *B. melitensis* and *B. abortus* in Xinjiang. Therefore, 8 isolation s of *Brucella* were to be a further analysis by AMOS-PCR. The results showed that

*B. abortus* was amplified the specify fragment of 498 bp, while *B. melitensis* was 731bp. The 8 strains of *Brucella* from 4 raw milk samples were amplified the specify fragment of 731 bp (Fig.3). It suggested that *B. melitensis* was the pathogen causing of brucellosis outbreaks in cattle.



**Fig. 3.** Identification of *Brucella* biotypes by AMOS-PCR

As the typing of isolated strains for epidemiological studies can help to back-trace an infection source, a definitive diagnosis for brucellosis were examined by biochemical test. In order to confirm the suspected link between cattle

and human strains, randomly selected 2 strains from 8 isolation strains were submitted to the Laboratory of Chinese center for disease control and prevention using biochemical test. Results showed they were *B.melitensis* biovar 3(Tab.1).

**Table 1.** The results of identification of isolated *Brucella* strains by biochemical test from raw milk

Identification of biotypes by biochemical experiments		Isolationof strains		The standard strains of <i>Brucella</i>			
		1	2	<i>B.melitensis</i> 16M	<i>B.melitensis</i> 63/9	<i>B.melitensis</i> Ether	
CO <sub>2</sub> requirments		-	-	-	-	-	
H <sub>2</sub> S production		-	-	-	-	-	
Dyeinhibition	Thionin (W/V)	1/25000	-	-	-	-	
		1/50000	+	+	+	+	+
	Rosein(W/V)	1/100000	+	+	+	+	+
		1/50000	+	+	+	+	+
Agglutination with mono- specific antisera	A	+	+	-	-	+	
	M	+	+	+	-	+	
	R	-	-	-	-	-	
biotype		<i>B.melitensis</i> biovar 3	<i>B.melitensis</i> biovar 3	<i>B.melitensis</i> biovar 1	<i>B.melitensis</i> biovar 2	<i>B.melitensis</i> biovar 3	

Notes: + is a positive ; - is a negative.

In several countries *B. melitensis*, causative agent of small ruminant brucellosis, is acquiring an increasing importance in bovine as an emergent pathogen (Corbel, 1997; Kahler, 2000;

Samaha *et al.*, 2008). Control of *B. melitensis* infection in cattle is impeded due to difficulties on the implementation of vaccination strategies (Denes, 1997, Vemulapalli *et al.*, 2004).To our

knowledge, its usefulness to control *B. melitensis* outbreaks in cattle in developed countries has not been fully assessed. Thus, slaughtering of all exposed animals is one of the available options to control a *B. melitensis* outbreak in a cattle herd. Alternatively, if the infection level is low and the outbreak is detected at an early stage, the disease might be controlled and eventually eradicated through the implementation of very strict management procedures (Julio *et al.* 2011).

*B. melitensis* infection in cattle poses a serious problem to both farmers and veterinarians, because of the lack of information regarding several aspects of its epidemiology, such as cattle -to-cattle transmission or within-herd persistence of infection (Bercovich, 1998). It is necessary to isolate and identify the etiologic agents to address these problems. In the study, it was allowed for isolation of pathogeny from raw milk and identification of the most likely source of infection by combination with AMOS-PCR and biochemical test. Depending on infection level (about 3.0%) and no brucellosis vaccination performed on the area, test-and-slaughter strategies were applied in the farm. The paper described the management of the outbreak of brucellosis due to *B. melitensis* in the farm.

It was taken several key measures for farms management system to control the outbreak. Firstly, immediate control measures adopted when the first cases were reported allowed. Culture and molecular characterization of the etiological agent from host species involved confirmed the possible origin of the outbreak. Positive animals were slaughtered and special care was taken to avoid any contact with these animals in order to minimize the risk of spreading the infection. Handling of these high risk animals by human workers was performed with extreme care. Secondly, Measures adopted on the farm were based on more than one type of technique to detect positive brucellosis, such as serum agglutination test (SAT), indirect enzyme-linked immunosorbent assay (iELISA), milk ring test (MRT) and the species-specific PCR assay. Thirdly, for sanitary and socio-economical reasons, and all animals in the farm were culled 120 days after detection of the outbreak. All animals were tested every 30 days using SAT, iELISA and MRT, with immediate removal of positive reactors. Especially, biosecurity measures were maximized

regarding handling of pregnant animals, increasing frequency of testing, disinfection of all material in contact with positive reactors. All cows calved in the farm in a separated area and special care to the discarded materials. The heifers were tested eight and 30 days post-birth to maximize the possibilities of detecting remaining infected animals after calving, in order to overcome difficulties in the diagnosis of *Brucella* infections in primiparous heifers, especially difficult to detect before calving. Finally, there was no a cattle to enter the farm and moved out to another farm during 2011-2013 year. And all animals in this farm were also subjected to regular testing for more than one year. Therefore, it was controlled the outbreak and eradicated brucellosis by above key actions in three years.

In conclusion, the characterization of species and biovars of *Brucella* field strains isolated in outbreaks is essential to understanding the disease epidemiology, to trace sources of infection and thus to design effective control strategies for brucellosis. Isolation *Brucella* from milk sample of dairy cattle could be a valuable diagnostic or screening test for herds with Brucellosis. On the other hand, it was necessary to notice that dairy products remain a health threat for food to people. If the infection level is low or at an early stage of the disease, the test-and-slaughter policy would be a reasonable strategy for the control of *B. melitensis* infection in cattle. Eventually, strict biosecurity measures can help to eradicate the disease from infected herds within a short term.

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#### REFERENCES

1. Bercovich, Z. Maintenance of *Brucella abortus*-free herds: a review with emphasis on the epidemiology and the problems in diagnosing brucellosis in areas of low prevalence. *Vet.*



- Q., 1998; **20**: 81-88.
2. Bricker, B.J. PCR as a diagnostic tool for brucellosis. *Vet Microbio*, 2002; **90**: 435-446.
  3. Corbel, M.J. Brucellosis: an overview. *Emerg Infect Dis.*, 1997; **3**: 213-221.
  4. Curro, V., Marineo, S., Vicari D., Galuppo, L., Galluzzo, P., Nifosi, D., Pugliese, M., Migliazzo, A., Torina, A. and Caracappa, S. The isolation of *Brucella* spp. from sheep and goat farms in Sicily. *Small Ruminant Research*, 2012; **106** (Suppl): S2-S5.
  5. Cutler, S.J., Whatmore, A.M. and Commander, N.J. Brucellosis - new aspects of an old disease. *J.App.Micro.*, 2005; **98**: 1270-1281.
  6. Denes, B. Serological findings obtained in cattle herds immunised with the *Brucella melitensis* Rev.1 and the *B. abortus* B19 vaccine in Mongolia. *Acta.Vet. Hung*, 1997; **45**: 33-43.
  7. Donev, D.M. Brucellosis as priority public health challenge in south eastern European countries. *Croat Med. J.*, 2010; **51**: 283-284.
  8. Julio, A., Jose, L.S., Sáez, B., Nerea G., Carles, S., Marta P.S., Sergio, G., Maria J. O., Josep, G., Lucio, C., Fulgencio, G., Joaquín G. and Lucas D. Management of an outbreak of brucellosis due to *B. melitensis* in dairy cattle in Spain. *Research in Vet. Sci.*, 2011; **90**: 208-211.
  9. Kahler, S.C. *Brucella melitensis* infection discovered in cattle for first time, goats also infected. *J. Am. Vet. Med. Assoc.*, 2000; **216**: 648.
  10. Karyn, A. H., Marine, R., Archil, N., Paata, I., Mo, S. The human-animal interface of domestic livestock management and production and its relationship to brucellosis in the country of Georgia 2010: A rapid assessment analysis. *Pre. Vet. Med.*, 2012; **105**: 10-16.
  11. Li, S.J., Wang, Y. and Chen, H. Isolation and Identification of *Brucella melitensis* firstly isolated from goat in Guizhou province. *Chinese J. Zoonos.*, 2011; **27**: 515-518.
  12. Moon, H., Dong-Hee, C., Sung, K., Yun-Sang, C., In-Yeong, H., You-Chan, B., Hachung, Y., Young-Ran, H., Suk-Chan, J. and Han Sang, Y. The development of a selective medium for the *Brucella abortus* strains and its comparison with the currently recommended and used medium. *Diag. Microbio and Infec. Dis.*, 2010; **67**: 15-21.
  13. Osterman, B., Moriyon, I. International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of *Brucella*. *Inter. J. Sys. Evol. Microbio.*, 2006; **56**: 1173-1175.
  14. Paschos, A., Patey, G., Sivanesan, D., Gao, C., Bayliss, R., Waksman, G., O'Callaghan, D. and Baron, C. Dimerization and interactions of *Brucella suis* VirB8 with VirB4 and VirB10 are required for its biological activity. *Proc. Natl. Acad. Sci. USA.*, 2006; **103**: 7252-7257.
  15. Samaha, H., Al-Rowaily, M., Khoudair, R.M. and Ashour, H.M. Multicenter study of Brucellosis in Egypt. *Emerg. Infect. Dis.*, 2008; **14**: 1916-1918.
  16. Seleem, M.N., Boyle, S.M. and Sriranganathan, N. Brucellosis: a reemerging zoonosis. *Vet. Microbiol.*, 2010; **140**: 392-398.
  17. Valderas, M.W. and Roop, R.M. *Brucella* and Bioterrorism. In: Anderson, B., Friedman, H., Bendinelli, M. (Eds.), *Microorganisms and Bioterrorism. Springer Verlag.*, 2006; 139-153.
  18. Vemulapalli, R., Contreras, A., Sanakkayala, N., Sriranganathan, N., Boyle, S.M. and Schurig, G.G. Enhanced efficacy of recombinant *Brucella abortus* RB51 vaccines against *B. melitensis* infection in mice. *Vet. Microbiol.*, 2004; **102**: 237-245.
  19. Wang, Y.Z., Chen, C.F. and Cui, B.Y. Isolation and identification of *Brucella melitensis* biovar 3. *Chinese J. Pre. Vet. Med.*, 2007; **29**: 753-755.
  20. Whatmore, A.M. Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. *Infect. Genet. E.*, 2009; **9**: 1168-1184.
  21. Yi, X.P., Ma, X.J., Yan, J.H., Gu, W.X., Liu, L.Y., Ye, F. and Zhong, Q. Isolation and identification of *Brucella* in raw milk. *Chinese J. Pre. Vet. Med.*, 2013; **35**: 28-30.
  22. Zhong, Q., Yi, X.P., Li, B., Wu, D.L., Liu, L.Y., Ye, F., Yan, J.H., Li, J.P. and Wang, L.J. Study and application of polymerase chain reaction assay on *Brucella* identification. *Chinese J. Zoonos.*, 2011; **27**: 241-245.
  23. Zhong, Q., Fan, W.X., Wu, D.L., Cai, Y.F., He, Q.N., Rehemu, J., Dawuti, Gu, W.X., Zhao, B. and Tuerh. Establishment of method to detect the virulence factor of *Brucella*, the VirB8-PCR assay. *Chinese J Zoonos.*, 2008; **24**: 50-54.
  24. Zhong Z.J., Yu S., Wang X.C., Dong S.C., Xu J., Wang Y.F., Chen Z.L., Ren Z.H., Peng G.N., Human brucellosis in the People's Republic of China during 2005-2010. *Intern. J. Infect. Dis.* 2013; **17**: 289-292.