Journal of Animal and Veterinary Advances 12 (9): 948-953, 2013
ISSN: 1680-5593

Characterization of Brucella Isolates from an Outbreak of Brucellosis after a Field Practice Without Protective Measures

[1,2]Yuanzhi Wang, [3]Ke Zhang, [2]Yali Zhang, [2]Hui Wang, [1,2]Fei Guo,
[2]Lin Zhang, [3]Hui Zhang, [3]Lijuan CaoBuyun, [4]Cui, [5]Chengyao Li,
[1,2]Li Yuan, [1,2]Wanjiang Zhang, [6]Ze Xu and [1,2,3]Chuangfu Chen
[1]Ministry of Education Key Laboratory for the High Incidence of Local and National Xinjiang,
[2]School of Medicine, [3]School of Animal Science and Technology,
Shihezi University, 832003 Shihezi, Xinjiang, P.R. China
[4]State Key Laboratory for Infectious Disease Prevention and Control,
Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases,
National Institute for Communicable Disease Control and Prevention, 102206 Beijing, P.R. China
[5]Department of Transfusion Medicine, School of Biotechnology,
Southern Medical University, 510515 Guangzhou, P.R. China
[6]Shanghai Center For Bioinformation Technology,
200235 Keyuan Road 1278, Shanghai Pudong New Area, Shanghai, P.R. China

Abstract: An outbreak of brucellosis occurred in students on field practice at sheep farm in 2005 at Shihezi, Xinjiang Province, the North-West of China. Five of 7 (71.4%) students were seropositive, showing titers ≥1:160 IU mL^{-1} in STAT and diagnosed as acute brucellosis with physical examination. To characterize Brucella isolates from the outbreak, the research including face to face investigation, Brucella isolation, multiple locus VNTR-16 analysis (MLVA-16) and genome sequencing were carried out. The investigation showed 42.5% (1,293/3,042) of ewes are sero-positive with RBPT and almost half of ewes aborted. Although, no bacteria were isolated from student blood samples, five individual colonies were isolated from aborted sheep fetuses and were identified as *B. melitensis* biovar 3 by conventional microbiological tests. MLVA-16 typing indicated that the isolates were clustered in the East Mediterranean with genotype 42. They were similar to wild strains from Guangdong in 2008 and Inner Mongolia in 1994 and 1995. They were most close to strain bru0261 from Pakistan student studying in Germany. Genome sequence and phylogenomic tree showed that pathogen in this study was close to Chinese wild and vaccine strains such as *B. melitensis* M28, M5-90. Researchers first report pathogens isolated in 1980 and 2005 are genotype 42 containing novel MLVA-16 patterns (1-5-3-13-2-2-3-2-4-20-8-8-4-3-7-7) compared to that both in China and other countries.

Key words: *B. melitensis*, outbreak, characterization, isolate, biovar

INTRODUCTION

Brucellosis is a worldwide zoonotic disease caused by Brucella which infects a broad range of mammals including domestic and wild animals as well as humans (Corbel, 1997; Haque *et al*., 2011). In China, approximately 30,000 human cases are reported annually over the past 5 years (Jiang *et al*., 2011). Since 1950, *Brucella melitensis*, one of *Brucella* genus has been the predominant species associated with human brucellosis in China (Jiang *et al*., 2010, 2011).

Xinjiang, the North-Western part of China, an area covering 1,600,000 km^2 where livestock is one of the most important resources for the regional economy is one of the areas with high prevalence of brucellosis (Wu *et al*., 2013). In 2005, a rare human outbreak of brucellosis occurred in college students who carried on a field study at a sheep farm. In order to determine transmission mode of *B. melitensis* from infected ewes to college students and characterize the pathogen, a face to face investigation, bacterial isolation, MLVA-16 typing and genome sequencing were carried out.

Corresponding Author: Chuangfu Chen, Ministry of Education Key Laboratory for the High Incidence of Local and National Xinjiang, Shihezi University, 832003 Shihezi, Xinjiang, P.R. China

MATERIALS AND METHODS

Epidemiologic investigation: In middle June 2005, two of seven students participated in a field study at a sheep farm complained of anorexia, fatigue and fever (>38°C). They suspected they might be infected with Brucella because of closely contacting with infected sheep in practical research. All seven students were examined by the Rose Bengal Plate Test (RBPT) and the Standard Tube Agglutination Test (STAT) at the local Center for Disease Control and Prevention (CDC). Five students were seropositive for *B. melitensis* ($\geq$1:160 IU mL^{-1}) and spleenmega by imaging examination. They were diagnosed as acute brucellosis. An epidemiologic survey was conducted by the face to face interview.

Microbiologic identification: To identify the pathogen involved in these human cases, bacterial isolation and identification were carried out from aborted sheep fetuses and students' blood samples. The samples were streaked onto the sheep whole blood agar media. The plates were incubated in a 10% CO_2 incubator at 37°C. All colonies were incubated for 12, 24 or 82 h and then transferred to duplicate plates and cultured again. The five pure colonies suspicious for Brucella with red acid-fast coccobacilli against a blue background by the modified Ziehl-Neelsen staining method (Foster *et al*., 1996) were sent to the Chinese Center for Disease Control and Prevention (CDC) for further conventional microbiological tests (Alton *et al*., 1975).

MLVA-16 typing: To determine the phylogenic relation between the five isolates in 2005 and other bacteria including domestic strains described previously by Jiang *et al*. (2010) abroad 529 worldwide strains registered with the MVLA bank database and Brucella strain 80/23 which is *B. melitensis* biovar 3 and was isolated from ram semen in Shihezi in 1980, all isolates were investigated by MLVA-16 typing described before (Al Dahouk *et al*., 2007; Le Fleche *et al*., 2006).

Genomic sequencing: To further characterize the isolates, the whole genome of Brucella strain 05/43 (also abbreviated as strain 043_1) was sequenced. The genomic DNA of *Brucella melitensis* 043 was isolated and sequenced by the whole genome shotgun strategy using Illumina Hiseq 2000 before constructing 500 bp library. The coverage was more than 100 fold and the paired-end reads (read1, 1-90 bp; read2, 1-70 bp) were assembled by SOAPdenovo 1.05 (Wang *et al*., 2010) (K = 39). Open Reading Frames (ORFs) were predicted by Glimmer 3.0 (Delcher *et al*., 2007). Furthermore, with the help of the genome database of 50 Brucella strains including 12 *B. melitensis*, 12 *B. abortus*, 9 Brucella strains from marine mammals, 7 *B. suis*, 2 *B. Canis*, *B. microti* CCM4915, *B. neotomae* 5K33, *B. ovis* 63/290 and *B.* sp. NVSL-0027, *B.* sp. BO1, *B.* sp. BO2, *B.* sp. NF 2653, *B.* sp. 83/13, the phylogenomic tree was built according to the methods from Audic *et al*. (2011). The multiple 2537 alignments were concatenated into a single alignment of 2226048 bases (about 2/3 of the Brucella genome). At last, the Phylogenetic tree was built by MEGA 5 (Tamura *et al*., 2011) using ClustalW 2.0 (Thompson *et al*., 2002) (Parameters Default) maximum likelihood tree.

RESULTS AND DISCUSSION

Investigation on the outbreak of brucellosis: In early March 2005, seven college students went to a sheep farm for a field study. The major task for the students was to quarantine 3,042 ewes in collaboration with two sheep farm workers. For the purpose of the RBPT analysis, the whole blood samples were collected from 3,042 sheep via the jugular vein with No. 12 gauge needles and Cap-Transport Tubes (Cat. No. BT888-SR, Brussels, Belgium) without any protective measure. Because the students and farm workers did not use vacutainer tubes, it is likely that some blood containing Brucella splashed onto their hands and faces, leading to an attendant risk of infection. In addition, the president of the sheep farm hid the fact that almost half of ewe had experienced abortion about a month ago before the students went for field practice. Subsequently, they found 42.5% (1,293/3,042) of ewes are sero-positive with RBPT. In middle June 2005, two of the seven students complained of anorexia, fatigue and fever. All the students were tested by RBPT and STAT at Xinjiang CDC. The highest STAT titer was 1:800 IU mL^{-1} in a student with severe arthralgia. During the students' hospitalization, a female sheep farm worker who had worked together with the students also exhibited fever and malaise and was diagnosed with acute brucellosis too.

The results of conventional microbiological tests: No bacteria were isolated from patient blood samples due to antibiotic therapy. Five individual colonies suspected to be Brucella were isolated from aborted sheep fetuses and all identified as *B. melitensis* biovar 3 (Table 1).

The molecular features of Brucella isolates: MLVA-16 typing revealed that same to strain 80/23, the five isolates in 2005 contained 16 identical loci and strain 05/43 was one of the five isolates. These strains clustered in the

East Mediterranean with genotype 42 (1-5-3-13-2-2-3-2) (Jiang *et al*., 2011). Compared with domestic Brucella isolates described previously by Jiang *et al.* (2011), strain 05/43, its MLVA-16 type (1-5-3-13-2-2-3-2-4-20-8-8-4-3-7-7) was similar to three strains including strain GD08-3 (Guangdong in 2008), NM94-0705 (Inner Mongolia in 1994) and NM95-1462 (Inner Mongolia in 1995) (Jiang *et al*., 2011) which harbored the same MLVA-16 type (1-5-3-13-2-2-3-2-4-20-8-5-4-3-7-7) and there was a Single-Locus Variant (SLV) at the bruce04 locus between strain 05/43 and GD08-3. Compared to abroad 529 worldwide Brucella isolates registered with the MVLA bank database, although, strain 05/43 was most close to the *B. melitensis* strain bru0261 from Pakistan student studying in Germany (Fig. 1), its MLVA-16 type (1-5-3-13-2-2-3-2-4-20-8-8-4-3-5-7) and there was a SLV at the bruce16 locus between strain 05/43 and bru0261.

Genome and genome analysis: The draft genome sequences of *Brucella melitensis* 043 was about 3.29 Mb.

Table 1: Biochemical features of wild *Brucella* sp. strains

						Standard strains		
Original No.	05/02	05/32	05/24	05/43	05/48	16M	544A	1330
Isolation from host	Sheep	Sheep	Sheep	Sheep	Sheep			
CO_2 requirement	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	+	++	+++
Acriflavin	-	-	-	-	-	-	-	-
Dye inhibition								
Thionin	+	+	+	+	+	+	-	+
Basic fuchsin	+	+	+	+	+	+	+	-
Lysitic test of phage[c]								
Tb RTD[b]	-	-	-	-	-	-	+	-
Tb[c]10[d] RTD	-	-	-	-	-	-	+	+
Iz	+	+	+	+	+			
BK2	+	+	+	+	+	+	+	+
Agglutination test of serum								
A	+	+	+	+	+	-	+	+
M	+	+	+	+	+	+	-	-
R	-	-	-	-	-	-	-	-
Identical results								
Species	*B. mel*[d]	*B. mel*	*B. mel*	*B. mel*	*B. mel*	*B. mel*	*B. abo*[e]	*B. suis*[f]
Biovar	3	3	3	3	3	1	1	1

[a]Dye concentration of Brucella agar medium (20 μg mL^{-1}; 1:50000); [b]RTD: The rate of Routine Test Dilution (the highest dilution rate at which the Tb phage completely lyses *Brucella* sp.); [c]Tb: Tbilisi; Iz: Izanagar; BK2: Berkeley; A: A mono-specific serum; M: M mono-specific serum; R: R mono-specific serum; [d]*Brucella melitensis*; [e]*Brucella abortus*; [f]*Brucella suis*

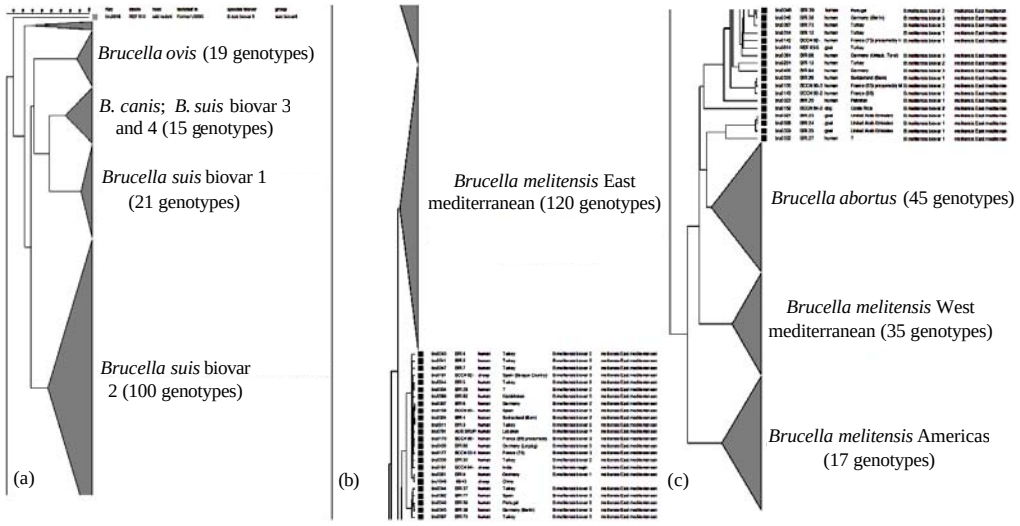

Fig. 1: Condensed dendrogram of clustered MLVA-16 genotypes obtained from 530 Brucella isolates (including Brucella 05/43) and corresponding to genotypes. This figure complies with data from MVLA database

Assemble of the genome yielded 20 scaffolds and 480 contigs. A total of 3436 ORFs were predicted. The average length of ORFs was 823 bp. All predicted ORFs accounted for 86.12% of the whole genome sequence of *Brucella melitensis* 043 (Sequenced, assembled and annotated by Shenzhen BGI in China).

Phylogenomic tree showed that strain 05/43 was more close to Chinese wild or vaccine Brucella strains such as *B. melitensis* M111 vaccine strain (Accession No. AFFB00000000), *B. melitensis* NI wild strain (Accession No. CP002931, CP002932), *B. melitensis* M28 (Accession No. CP002459, CP002460) and its derivative strains including M28-12 wild strain (Accession No. AFFA00000000), M5 and M5-90 vaccine strains (Accession No. AFEZ00000000, CP001851 and CP001852) (Fig. 2).

Brucellosis is a highly contagious zoonotic disease. Almost all of human brucelosis cases are directly or indirectly associated with contacting the infected animals (Luna-Martinez and Mejia-Teran, 2002). Furthermore, *B. melitensis* is highly contagious, laboratory workers could be infected by aerozolization (Fiori *et al*., 2000; Robichaud *et al*., 2004; Staszkiewicz *et al*., 1991; Zervos and Bostic, 1997). Brucellosis has been an occupational risk for farmers, veterinarians and employees in the livestock business (Young, 1995). Nonoccupational sources of infection include consumption of fresh, unpasteurized raw fresh milk and milk products (Palencia and Sinovas, 1997). In human, brucellosis can cause multisystemic disease with varying spectrum of symptoms. In this investigation, researchers found that this sheep farm was newly built and all the ewes were collected from markets. To quarantine these ewes, the president of this sheep farm offered the practice field for college students. When 7 college students found the sero-positive rate was 42.5%, they were informed that about 50% of these ewes aborted 4 months ago. To ensure whether they were infected, serological tests and imaging examination were carried out. It was found five of seven students had been infected with Brucella combined with the bacterial tests. The report highlights the importance of personal protection from brucellosis when contacting with Brucella-infected or animals suspected of infection in the course of their occupational duties.

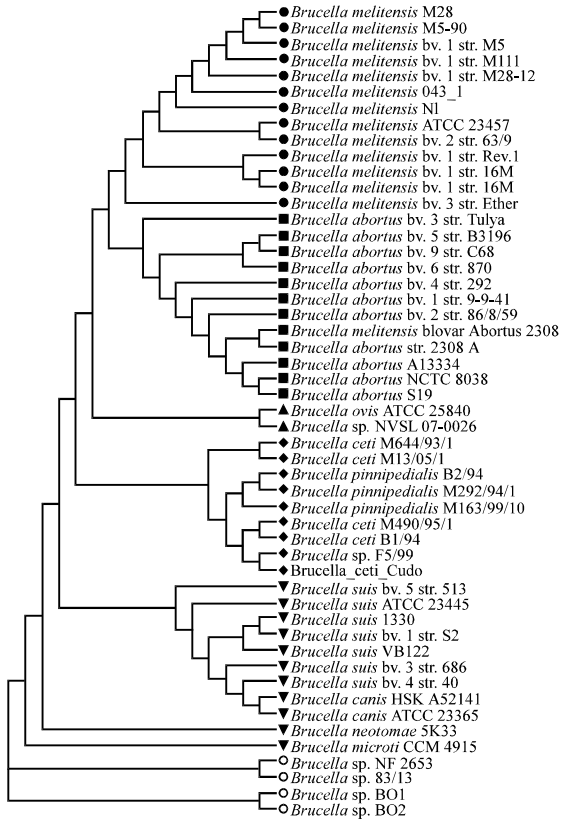

Fig. 2: Phylogenic tree is based on 51 Brucella whole genome sequences

MLVA typing is popularly used to study evolutionary model of pathogens or trace back the source of infection such as *Streptococcus agalactiae*, *Staphylococcus aureus*, *Yersinia pestis* and (Haguenoer *et al*., 2011; Riehm *et al*., 2012; Sobral *et al*., 2012). In this study, it was found there were identical MLVA-16 data between Brucella 05/43 strain and 80/23 strain isolated in 1980 and 2005, respectively. This result suggested that isolated 05/43 strain should be a local pathogen responsible for brucellosis outbreak and researchers speculate that they might settle in Shihezi at least 25 years. In addition, as far as the regional distribution of Brucella 05/53 strain and its close isolates is concerned, they are located between North latitude 30°-65° around East Mediteranean region (Fig. 3). It was suggested that distribution of *B. melitensis* might be influenced by the geographical factors.

With the genome sequencing technology development, new sight into biology has been available. To investigate clearly the evolution of the isolates, the genome of *B. melitensis* 043 was sequenced and phylogenomic tree was built. It was clear that strain 043 was clustered in the clade of *B. melitensis* and it was close to wild and vaccine *B. melitensis* strains in China. The finding suggested that vaccine strains from local wild isolates may provide more protection compared with abroad vaccines because of more close relationship.

Fig. 3: *B. melitensis* 05/43 strain and its close homologs identified in regions of Northern latitude 30°-65°

CONCLUSION

Here, researchers describe the tragic case of outbreak from a brucellosis involved in 5 college students and a sheep farm worker in Shihezi, Xinjiang. Researchers first report pathogens isolated in 1980 and 2005 are genotype 42 containing novel MLVA-16 patterns compared to that both in China and other countries. The pathogen such as strain 05/43 is more close to Chinese wild or vaccine Brucella strains. The whole-genome shotgun sequences (contigs) of *B. melitensis* 05/43 have been submitted at GenBank under the accession numbers: ANOD00000000.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Basic Research Program of China (2010CB530203), the National Science and Technology Pillar Program (2013BAI05B05) and the National Nature Science Foundation of China (31060334 and 31260596). Researchers would thank Philippe Le Fleche (University of Paris XI, Orsay, France) for his advice on the MLVA-16 typing. Yuanzhi Wang and Ke Zhang contributed equally to this research.

REFERENCES

Al Dahouk, S., P. Le Fleche, K. Nockler, I. Jacques and M. Grayon *et al*., 2007. Evaluation of *Brucella* MLVA typing for human brucellosis. J. Microbiol. Methods, 69: 137-145.

Alton, G.G., L.M. Jones and D.E. Pietz, 1975. Laboratory Techniques in Brucellosis (World Health Organization Monograph Series No. 55). 2nd Edn., WHO, Geneva, Switzerland, ISBN-13: 9789241400558, Pages: 163.

Audic, S., M. Lescot, J.M. Claverie, A. Cloeckaert and M.S. Zygmunt, 2011. The genome sequence of *Brucella pinnipedialis* B2/94 sheds light on the evolutionary history of the genus *Brucella*. BMC Evol. Biol., Vol. 11. 10.1186/1471-2148-11-200

Corbel, M.J., 1997. Brucellosis: An overview. Emerg. Infect. Dis., 3: 213-221.

Delcher, A.L., K.A. Bratke, E.C. Powers and S.L. Salzberg, 2007. Identifying bacterial genes and endosymbiont DNA with glimmer. Bioinformatics, 23: 673-679.

Fiori, P.L., S. Mastrandrea, P. Rappelli and P. Cappuccinelli, 2000. *Brucella abortus* infection acquired in microbiology laboratories. J. Clin. Microbiol., 38: 2005-2006.

Foster, G., K.L. Jahans, R.J. Reid and H.M. Ross, 1996. Isolation of *Brucella* species from cetaceans, seals and an otter. Vet. Rec., 138: 583-586.

Haguenoer, E., G. Baty, C. Pourcel, M.F. Lartigue and A.S. Domelier *et al*., 2011. A multi locus variable number of tandem repeat analysis (MLVA) scheme for *Streptococcus agalactiae* genotyping. BMC Microbiol, Vol. 11. 10.1186/1471-2180-11-171.

Haque, N., M.S. Bari, M.A. Hossain, N. Muhammad and S. Ahmed *et al*., 2011. An overview of Brucellosis. Mymensingh Med. J., 20: 742-747.

Jiang, H., L.L. Mao, H.Y. Zhao, L.Y. Li, D.R. Piao, W.Q. Yao and B.Y. Cui, 2010. MLVA typing and antibiotic susceptibility of *Brucella* human isolates from Liaoning, China. Trans. R. Soc. Trop. Med. Hygiene, 104: 796-800.

Jiang, H., M. Fan, J. Chen, J. Mi and R. Yu *et al*., 2011. MLVA genotyping of Chinese human *Brucella melitensis* biovar 1, 2 and 3 isolates. BMC Microbiol., Vol. 11. 10.1186/1471-2180-11-256.

Le Fleche, P., I. Jacques, M. Grayon, S. Al Dahouk and P. Bouchon *et al*., 2006. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiol., Vol. 6. 10.1186/1471-2180-6-9.

Luna-Martinez, J.E. and C. Mejia-Teran, 2002. Brucellosis in Mexico: Current status and trends. Vet. Microbiol., 90: 19-30.

Palencia, R. and M.I. Sinovas, 1997. Epidemiology and clinical manifestations of migraine in children. An. Esp. Pediatr., 2: 181-183.

Riehm, J.M., G. Vergnaud, D. Kiefer, T. Damdindorj and O. Dashdavaa *et al*., 2012. *Yersinia pestis* lineages in Mongolia. PLoS ONE, Vol. 7. 10.1371/journal.pone.0030624.

Robichaud, S., M. Libman, M. Behr and E. Rubin, 2004. Prevention of laboratory-acquired brucellosis. Clin. Infect. Dis., 15: e119-e122.

Sobral, D., S. Schwarz, D. Bergonier, A. Brisabois and A.T. Fessler *et al*., 2012. High throughput multiple locus variable number of tandem repeat analysis (MLVA) of *Staphylococcus aureus* from human, animal and food sources. PloS ONE, Vol. 7. 10.1371/journal.pone.0033967.

Staszkiewicz, J., C.M. Lewis, J. Colville, M. Zervos and J. Band, 1991. Outbreak of *Brucella melitensis* among microbiology laboratory workers in a community hospital. J. Clin. Microbiol., 29: 287-290.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Mol. Biol. Evol., 28: 2731-2739.

Thompson, J.D., T.J. Gibson and D.G. Higgins, 2002. Multiple Sequence Alignment using ClustalW and ClustalX. In: Current Protocols in Bioinformatics, Baxevanis, A.D. (Ed.). Vol. 2, Unit 2.3, John Wiley and Sons Inc., New York, USA., ISBN-13: 9780471250937, pp: 1-22.

Wang, J., J. Shao, J. Li, S. Gao, J. Du, G. Cong and H. Chang, 2010. Fusion expression of Asia I type FMDV neutralizing epitope with heavy chain constant region of sheep IgG and the assessment of its immunogenicity. Sheng Wu Gong Cheng Xue Bao, 26: 454-461.

Wu, G., C. Yang, J. Li, N. Liu, W. Yao, R. Zhang and Z. Lin, 2013. Prevalence study of brucellosis among high-risk people in Xinjiang region, China. Microbiol. Discovery. 10.7243/2052-6180-1-2.

Young, E.J., 1995. An overview of human brucellosis. Clin. Infect. Dis., 21: 283-289.

Zervos, M.J. and G. Bostic, 1997. Exposure to *Brucella* in the laboratory. Lancet, 349: 651-651.