American Journal of Biomedical and Life Sciences
2018; 6(3): 63-68
http://www.sciencepublishinggroup.com/j/ajbls
doi: 10.11648/j.ajbls.20180603.15
ISSN: 2330-8818 (Print); ISSN: 2330-880X (Online)

An Epidemiologic Study on Brucellosis in the Vicinity of Hohhot in China

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To cite this article:
Xiaohui Wang, Dexi Li, Lidao Bao, Ruilian Ma, Sha Li. An Epidemiologic Study on Brucellosis in the Vicinity of Hohhot in China. *American Journal of Biomedical and Life Sciences*. Vol. 6, No. 3, 2018, pp. 63-68. doi: 10.11648/j.ajbls.20180603.15

Received: May 9, 2018; **Accepted**: July 20, 2018; **Published**: August 13, 2018

Abstract: In the past five years, the epidemic situation of brucellosis in Hohhot, Inner Mongolia, has been aggravated. The current diagnostic technology has low specificity. Through this topic, we can find a more convenient, safer, more specific and sensitive method for preparation. Better antigens are used for early rapid diagnosis of Brucellosis. Molecular biology techniques were used to design primers based on the published bp26 gene of Brucella from Gen-Bank. The DNA of Brucella isolates was extracted, PCR amplified and sequenced, and the bp26 gene sequence of the reference strain was identical to that of the reference strain. By comparing and analyzing the origins, the bp26 gene was amplified and compared with sheep, cattle, pigs, and sheep. In 2010, random surveys were conducted among 11401 people in 5331 farmers around Hohhot. The infection rate of Brucellosis was 3.82%, and 139059 of sheep were sampled in township and townships, and the positive rate was 2.82%. The results of bp26 gene sequence showed that the isolates of this experiment were 99.9% homologous to Brucella species 16M and Brucella species C68. The isolates were Brucella species 1320, Epididymis oryzae 280 and cattle. The homology of the Brucella strain S18 was 99.8%, 99.8%, and 98.9%, respectively. According to the phylogenetic tree analysis, the bp26 genes of Brucella strains of different strains are close, indicating that bp26 gene is very conservative. The homologous genes obtained by gene amplification technology can be used to detect br26 disease using bp26 protein. Other possible interferences can be ruled out and the diagnosis of Brucella can be promptly made. The culture and control of virulent strains of Brucella can be avoided. This experiment laid the foundation for the establishment of a new detection method using recombinant bp26 protein.

Keywords: Brucellosis, Early Diagnosis, bp26 Gene, PCR

1. Introduction

Brucellosis is a zoonotic infectious disease. Its clinical characteristics are mainly manifested with fever, sweating, fatigue, joint and muscle pain, changes in the reproductive system, and symptoms in the nervous system, digestive system and cardiovascular system for some patients [1]. Brucellosis not only harms people's health, but also greatly affects their livelihood, including the demand for milk, meat, skin, hair and bone, etc., and it also exerts influence on the development of animal husbandry to a considerable extent, and the international trade and tourism as well.

According to the epidemiological investigation on the incidence of brucellosis in the surrounding areas of Hohhot City, Inner Mongolia Autonomous Region, P. R. China, the epidemic situation has been significantly aggravated over the past five years, therefore, timely and effective detection of brucellosis is extremely urgent. Currently, bacteriological detection is the gold standard for clinical brucellosis detection [2], however, this method can not be used in the early and rapid diagnosis as a result of its time and energy consuming feature. In the existing three common antigens selected by serological techniques, bacterial suspension, whose preparation is simple, has relatively high diagnostic value, but for its low specificity, the results assessment is hard to be quantified, which might be easy to cause missed diagnosis; LPS is a recognized substance that can stimulate the production of antibodies [3], but common antigenic components exist between it and other bacteria as well as the

same bacteria in different genera, which can not rule out the possibility of false positive results in test [4]; although its purity of purified protein derivative is higher than that of the former two, with small toxic and side effects and high specificity and sensitivity [5], the serological cross reaction with Yersinia type O9 can not be avoided. In addition, the preparation processes of the three antigens involve live bacteria culture, presenting certain risks of bio-safety; therefore, it is of great significance to finding an antigen which is easily to be prepared and safer with higher specificity and sensitivity for the early and rapid diagnosis of brucellosis [6]. In order to study the molecular biological characteristics of brucellosis in Hohhot City, the molecular biology methods were adopted in this test to design a pair of primers according to the bp26 gene sequence of brucellosis published the Gen-Bank, extract DNA from the Brucella isolates for PCR amplification and sequencing, and then make homology comparison with the bp26 gene sequences of the reference strains and analysis, for the purpose of amplifying bp26 gene [7], so as to lay a basis of molecular biology for the research on brucellosis control in Hohhot.

2. Materials and Methods

2.1. General Disease Onset Status

In 2010, we investigated key groups of brucellosis - 11401 people in 5331 raising households engaged in industries related to animal husbandry such as cultivation, animal epidemic prevention and animal products processing in 203 units in 13 villages and towns in the periphery of Hohhot City randomly by visiting farming and stockbreeding areas, issuing questionnaires and brucellosis detection and found that 435 people were infected with brucellosis, with an infection rate of 3.82% and the highest infection rate was up to 23.46% (Table 1).

The epidemic situation of brucellosis has been showing a sustained growth trend in recent years in this region, its incidence range almost involved all villages and towns, and this situation has shifted from occupational groups to the general public. There was a relatively large epidemic rise in 2009, 2010 and 2011, which increased by 90.0% in 2009 compared with 2008, 77.6% in 2010 compared with 2009, and 60.3% in 2011 compared with 2010. (The above data sourced from epidemic statistical statements).

Table 1. *Infection of brucellosis.*

Village	Unit No.	Raising household No.	Investigation No.	Infection No.	Infection rate/%
1	20	93	445	28	6.29
2	14	496	1102	29	2.63
3	11	338	730	4	0.55
4	11	357	831	0	0
5	12	1336	2679	36	1.34
6	18	477	770	26	3.38
7	20	152	618	145	23.46
8	19	123	335	11	3.28
9	10	354	693	18	2.60
10	13	237	762	47	6.17
11	19	555	814	12	1.47
12	21	441	920	43	4.67
13	15	372	702	36	5.13
Total	203	5331	11401	435	3.82

Disease onset of livestock

13,883 cattle in 3360 large-scale farms (raising households) and scattering raising households and 219,672 sheep in 4,624 large-scale farms (raising households) and scattering raising households in 12 villages and towns were investigated, with the survey scope in this region reaching 64.50%, and the survey on cattle and sheep accounted for 67.88% and 94.01% respectively of the total amount of livestock on hand (Table 2).

Table 2. *Infection status of cattle and sheep.*

Livestock	Scale	Total No.	Sample No.	Positive No.	Positive rate/%	Compared to the results in 1989
Cattle	Below 10	7948	4913	53	1.08	0.29
	Over 10	5935	4515	984	21.79	21
	Entire village	13883	9428	1037	10.99	10.2
Sheep	Below 100	82797	44956	1297	2.88	2.04
	Over 100	136875	94103	2625	2.78	1.93
	Entire village	219672	139059	3922	2.82	1.97

9,428 cattle were sampled from the townships in this region, of which 1037 were positive, with a positive rate of 10.99%. The result was 10.2 percentage points higher than the census result in 1988 (0.79%), showing an obvious rising trend. Moreover, for both livestock farms (raising households) with the scale of more than 10 and scattering raising households with the scale of less than 10, the infection rate of bovine brucellosis was significantly higher than the census result in 1989 (0.79%);

The infection rate of bovine brucellosis was 21.79% in the livestock farms (raising households) with the scale of more than 10, which was 0.29% higher than that in the scattering

raising households with the scale of less than 10. The sampling rates of both the scales were up to 75% and 62% respectively.

In the 139,059 sheep sampled in the villages and towns in this region, 3,922 were positive, which was 1.97 percentage points higher than the census result of 0.85% in 1989, showing an obvious upward trend. In addition, for both livestock farms (raising households) with the scale of more than 100 and scattering raising households with the scale of less than 100, the infection rate of ovine brucellosis was significantly higher than the result of the census in 1989 (0.85%).

The infection rate of ovine brucellosis was 2.78% in the livestock farms (raising households) with the scale of more than 100, lower than that in the scattering raising households with the scale of less than 100, which was 2.88%. The sampling rates of both the scales were up to 67% and 55% respectively.

2.2. Blood Test of Brucellosis in Helingeer County

Since brucellosis occurred between human and livestock in Helingeer County, Hohhot City in 2008, leaders at all levels have attached great importance to the purification of brucellosis among livestock. Over the past five years, we have been conducting blood test every year on livestock (cattle and sheep), and killing and purifying positive animals in a timely manner, and taking comprehensive measures of quarantine, immunization, killing and disinfection to curb the prevalence of brucellosis infection, which has yielded good results. The blood test report for brucellosis among livestock in Hohhot between 2008 and 2012 was as follows.

In 2008, 2,815 cattle were sampled in total, including 189 positive cattle with a positive rate of 6.71%; in 2009, 1789 in total, including 69 positive cattle with a positive rate of 3.85%; in 2010, 186 in total, including 4 positive cattle with a positive rate of 2.16%; in 2011, 388 in total, including 3 positive cattle with a positive rate of 0.89%; in 2012, 415 in total, including 1 positive cattle with a positive rate of 0.024%.

In 2008, 8,170 sheep were sampled in total, including 250 positive sheep with a positive rate of 3.06%; in 2009, 11,300 in total, including 10 positive sheep with a positive rate of 0.08%; in 2010, 13,800 in total, including 22 positive sheep with a positive rate of 0.16%; in 2009, 13,220 in total, including 2 positive sheep with a positive rate of 0.021%; in 2012, 18,200 in total, including 6 positive sheep with a positive rate of 0.036%.

The detection conditions are shown in Table 3 in detail, and the incidence of human brucellosis is listed in Table 4.

Table 3. *Livestock blood test report.*

		2008	2009	2010	2011	2012 (January-June)
Cattle	On hand No.	2910	2285	2950	3100	3140
	Blood test No.	2815	1789	185	388	415
	Positive No.	189	69	4	3	1
	Positive rate/%	6.71	3.85	2.16	0.89	0.024
Sheep	On hand No.	940 thousand	970 thousand	980 thousand	1.05 million	1.16 million
	Blood test No.	8170	11300	13800	13220	18200
	Positive No.	250	10	22	2	6
	Positive rate/%	3.06	0.08	0.16	0.021	0.036

Table 4. *Incidence of human brucellosis.*

	2008	2009	2010	2011	2012 (January-June)
Onset No.	40	36	16	15	4

Sequence analysis of brucellosis bp26

bp26 protein is a soluble outer peripheral plasma protein with strong immunogenicity that can be released from inside to outside of cells. The bp26 antigen can be detected in brucellosis infection of bovine, sheep, goat and human [8], and the accuracy rate can reach 90% or above by using bp26 protein as the antigen for detection [9]. According to the bp26 gene sequence published in the GenBank, the upstream primer p1 and downstream primer p2 should be designed on the both sides of its coding region, and the DNA of brucellosis is extracted for PCR amplification and sequencing [10]. As reported in the previous literatures [11], there are the following advantages with the recombinant bp26 protein instead of the crude protein extracted from brucellosis in its diagnosis: 1) bp26 is the active ingredient dissociating from brucella antigens that may interfere with diagnostic test [12]; 2) the recombinant bp26 protein is less time-consuming, but has high yield; 3) the culture and control of virulent strains of brucellosis can be avoided [13]. In this test, the bp26 gene of brucellosis isolates was amplified by PCR for comparative analysis of sequencing, which laid a foundation for the establishment of a new method for the detection by recombinant bp26 protein.

2.3. Bacteria Source

Inactivated ovine (Malta) brucellosis was offered by the Brucellosis Control Department, Research Center for Endemic Disease Control, Inner Mongolia Autonomous Region.

2.4. Regents and Kits

Low melting-point agarose gel, PCR reagent and DL-2 000 Marker were purchased from Sigma Company (USA), and Bacteria DNA Kit from Boster Biotechnology Co. Ltd.

2.5. Reference Gene Sequence

The reference strains and their GenBank accession numbers were as follows: B. suis 1320, AY166768.1; B. ovis 280, AY166769; B. abortus C68, Y166765; B. abortus S18, AY166766.1; B. melitensis 16M, U45996.

3. Methods

3.1. Primer Design and Synthesis

According to the published bp26 gene sequence, the upper and downstream primers and restriction sites were designed on both sides of the coding region, amplified fragment size expected to be 771 bp. The primer sequences were p1 5'-CGG-GAATTCATGTACACTCGTGCTAGC-3' for the upstream primer and p25'-CGCGGATCCTTACTTGAAATCAATTACG-3' for the downstream primer. The primers were synthesized by Boster Biotechnology Co., Ltd.

3.2. Preparation of DNA Template

The ovine (Malta) brucellosis used in this test was the inactivated bacterial suspension of isolated wild strains. The test steps are as follows: take 1 mL culture into 1.5 mL EP tube, and centrifuge for 5 min at 8,000 r/min and room temperature; remove supernatant, resuspend sediment in 1 mL TE (pH 8.0); add 6μL50 mg/mL lysozyme for 2 h at 37°C; then add 50 μL 2 mol/L NaCl, 110 μL 10% SDS and 3μL 20 mg/mL proteinase K for 3 h at 50°C or 37°C overnight (when the bacteria suspension should be transparent and viscous liquid); divide the suspension equally into two 1.5 mL EP tubes, add an equal volume of mixture with phenol: chloroform: isoamyl alcohol (25:24:1), mix and place it at room temperature for 5 to 10 min; centrifuge at 12,000 r/min for 10 min [twice extractions in total (Note to be careful in the extraction for the supernatant is very sticky. The best way is to cut off pipette tips.)]; add 0.6 time the volume of isopropanol, mix and place it at room temperature for 10 min; centrifuge at 12,000 r/min for 10 min; take and wash the sediment with 75% ethanol for the PCR template after dry in the air.

3.3. PCR Amplification

The reaction system (25 μL) included 12.5 μL Taqmix, 2μL template DNA, 0.75μL primer p1, 0.75μL primer p2 and 9 μL water. The PCR reaction parameters were 94°C for 4 min; 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, 30 cycles in total; extension at 72°C for 10 min.

5 μl PCR products were added to 1.0% agarose gel for electrophoresis at 100 V for 30 min. The results were observed by the gel imaging system. The PCR products were sent to Boster for sequencing.

3.4. Sequence Analysis

The DNA Star software was adopted for homologous comparison and analysis on the measured bp26 gene sequence and the bp26 gene sequences of reference strains and drawing the evolutionary tree.

4. Results

4.1. PCR Amplification

After the detection of agarose gel electrophoresis, the products were found clear bands at approximately 750 bp (Figure 1), with the size consistent with expectations.

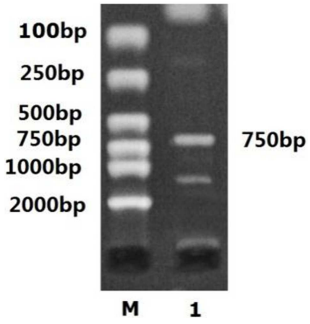

Figure 1. *PCR amplification of bp26. M: DL-2000 Marker; 1: isolated strain.*

4.2. Sequence Analysis of bp26

The PCR products were subjected to sequence determination, and the measured sequences were spliced by using computer software to obtain the nucleotide sequence of bp26 gene, which was 771 bp in length and contained a complete open reading frame (10～753 bp). The nucleoside sequences are shown in Table 5.

Table 5. *Nucleoside sequences of bp26.*

1	aggttcactc	cgtacgtacg	tttgcatgca	gcctagcaaa	acgtgggact	cagtcgtaac
61	gcatttactg	gcaaagctgt	tagcgtttac	caaacgtcag	ggttacagct	gctcccacgt
121	gycaattgtc	cgtttcacgg	acgtgaaacg	tgcaccacgt	acggttgcaa	cgtaaacgtc
181	aattcgctat	aaatctggca	tctcggacta	gaaagtccca	agtgtcccta	atctagtacg
241	gtgaacgtac	aaagtcacgt	ggcttacgta	cgatttcacg	cagtttcaca	gtagtacgtg
301	gtcaacgtta	acgtgcctgc	cgtagggctc	aagctccctg	ttgcaacgta	agtgaaacgt
361	gacaaacgct	ggtacgcgtg	caaacgtgta	aatcgtttac	atcgcctact	ggctcgaact
421	agctagctga	atgcgggact	aggtcgtagt	tattttacgg	agctgcccta	tgcgaaacgt
481	agggctgtac	aatcgtgcta	aggctagcc	acccctgta	accccgtaag	aagtgcgtag
541	agttgctccc	aaatcgtacg	atccgagtac	cctgagcaac	aattcgagct	acgccactg
601	ccggttagctg	atcgccgtag	gccctagcta	cgtacgttca	tagctacgta	acgtgtccgt
661	acatgctaat	ggatcggcta	aattggcacg	ctggactgac	agctaacgtc	agggtgacca
721	cgtagctagt	actgctgggt	gagtcgatcg	ccggtaggca	cgtcgtaaac	acgtaaggcg

4.3. Comparison Between bp26 and Reference Sequences

The sequence results showed that the homology of the isolates in this test with B. melitensis 16M and B. abortus C68 was 99.9%; 99.8%, 99.8% and 98.9% respectively with B. suis 1320, B. ovis 280 and B. abortus S18.

According to the analysis of phylogenetic tree, bp26 genes had close genetic relationship among the different strains of melitensis, proving that the bp26 gene was very conservative, which was entirely consistent with the expected results (Figure 2).

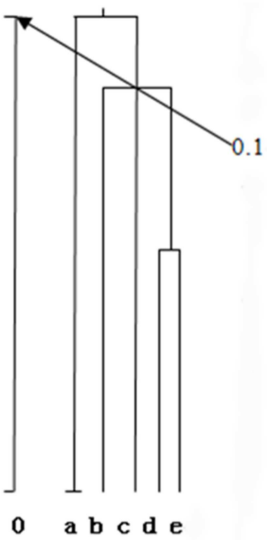

Figure 2. *Phylogenetic tree of bp26 genes. a: S18, C68; b: isolated strain; c: 280; d: 1320; e: 16M.*

5. Discussion

9,428 cattle were sampled from the villages and towns in the vicinities of Hohhot, of which 1037 were detected positive, with a positive rate of 10.99%. The result was 10.2 percentage points higher than the census result in 1988 (0.79%), showing a rising trend. Moreover, for both livestock farms (raising households) with the scale of more than 10 and scattering raising households with the scale of less than 10, the infection rate of bovine brucellosis was significantly higher than the census result in 1989 (0.79%); in the 139,059 sheep sampled, 3,922 were positive, which was 1.97 percentage points higher than the census result of 0.85% in 1989, showing an obvious upward trend. In addition, for both livestock farms (raising households) with the scale of more than 100 and scattering raising households with the scale of less than 100, the infection rate of ovine brucellosis was significantly higher than the result of the census in 1989 (0.85%).

According to the blood test results of brucellosis in Horinger County, Hohhot, the incidence rates of cattle brucellosis from January to June between 2008 and 2012 were 6.71, 3.85, 2.16, 0.89 and 0.024 respectively; those of sheep brucellosis were 3.06, 0.08, 0.16, 0.021 and 0.036 respectively; and the incidence numbers of human brucellosis were 40, 36, 16, 15 and 4 respectively. The declining trend of brucellosis incidence in this County was closely related to the blood test on livestock (cattle, sheep) every year, timely killing and cleansing positive domestic animals and the implementation of comprehensive measures of quarantine, immunization, killing and disinfection.

In this test, bp26 gene was successfully amplified by PCR, and comparative analysis was made between the measured bp26 gene sequence and that of the reference strains. The results indicated that the homology was 99.9% with B. melitensis 16M and B. abortus C68; 99.8%, 99.8% and 98.9% respectively with B. suis 1320, B. ovis 280 and B. abortus S18. The results of phylogenetic tree analysis also showed that the isolates in this test had close genetic relationship with the reference strains. The test results suggested that the bp26 gene was relatively conservative. The successful amplification of bp26 gene in this test laid a basis of molecular biology for the research of brucellosis control in Hohhot.

Currently, the prevention and control of brucellosis has not been incorporated into the scope of compulsory immunization in China, however, this disease has drawn high attention of the municipal party committee, municipal government and hygiene and animal husbandry agencies since the incidence of brucellosis between people and livestock in 2008, and great achievements have been made over the years of hard work. With the rapid development of the market economy and fast increasing circulation of animals and animal products, the prevention and control work is confronted with arduous tasks. Efforts slack off occasionally, and the prevention and control outcomes fail to be consolidated. Given all of that, the governments at all levels and all business sectors should attach great importance to this phenomenon.

In addition, for inadequate supervision, large trading volume of slaughter and animals products, combined with weak awareness of produced-area quarantine for some wholesalers, they are provided with the opportunity of evading quarantine, so the phenomena of not declaring quarantine after transfer have occurred from time to time. Moreover, some epidemic prevention personnel, with poor sense of responsibility, do not perform immunity according to the operating procedures, resulting in immune density not up to the standards [14].

There are some recommendations for the control, prevention and treatment of brucellosis: (1) to strengthen the publicity of basic knowledge of the prevention and control of brucellosis, especially the popularization of prevention knowledge to high-risk population, and ensure infected people to be effectively treated [15]. (2) To strengthen trainings for primary-level veterinary personnel, enabling them to master the simple and efficient diagnostic techniques of brucellosis among domestic animals. (3) To strengthen the quarantine supervision and gavage immunization [16], all transported susceptible livestock species must be immunized and quarantined according to the prescribed procedures. Susceptible livestock species should be conducted gavage immunization to ensure their antibody levels meet the requirements of national regulations. The supervision and

management on transported animals should be strengthened to curb the occurrence and spread of brucellosis from the source, so as to ensure public health and safety [17]. (4) All positive domestic animals should be conducted bio-safety disposal, and the contaminated sites, utensils and items must be strictly disinfected. (5) To strengthen personnel protection. Feeders should maintain regular physical examination annually, and leave the post and receive treatment immediately once found to be infected with brucellosis [18]. (6) To enhance effective coordination and cooperation between departments, strengthen the construction of professional epidemic prevention team, and mobilize more social resources to jointly cope with brucellosis; reinforce the supervision and management on animal health in accordance with the law, effectively strengthen quarantine and supervision, immune, harmless treatment of infectious mortalities and other measures to eliminate the source of infection among domestic animals with high responsibility [19].

6. Conclusion

At the same time, according to the sequence analysis test of brucellosis bp26 gene, the PCR results showed that the homologous genes can be obtained by gene amplification techniques for the detection of brucellosis by bp26 protein, which can rule out other possible interferences to quickly diagnose brucellosis, and can also avoid the culture and control of virulent strains of brucellosis. This test lays a foundation for the establishment of new detection method based on the recombinant bp26 protein.

Acknowledgements

This research is funded by the following projects: Youth Innovation Fund of Inner Mongolia Medical University (YKD2017QNCX051); the Major scientific research projects of Affiliated Hospital of Inner Mongolia Medical University (NYFY ZD 2017010); the Nature Science Foundation of Inner Mongolia Autonomous Region (2017MS0831 and 2018LH08032); 2017MS (LH) 0836 for Dexi Li), http://nsbr.nmkjt.gov.cn/index.aspx.

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