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Comparison and Analysis of Omp25 Genes of Nine *Brucella* Field Strains from Dairy Cows with Brucellosis in China

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Abstract: The full omp25 genes (642 bp) of 9 *Brucella* field strains from in 9 milk specimens sampled from dairy cows positive for brucellosis SAT on 9 dairy farms of different Provinces in China were molecularly characterized by PCR using the primers designed according to the published *B. abortus* omp25 gene sequence. Sequencing, comparison and analysis of the omp25 gene sequences cloned from the 9 *Brucella* field strains revealed a high degree of homology among their DNA sequences (>98%), but all the sequences had some variations and none of them was same as the published sequences. The result revealed that *Brucella* field strains were mutated at various degrees in the different regions of China. The phylogenetic tree of omp25 gene has revealed the sequences of the field strains from Gansu and Shaanxi Provinces were closed to the *B. suis* (99.7 and 99.8% homology). Therefore, it is presumed that cows were affected with *B. suis*, which has been reported in China.

Key words: *Brucella suis*, omp25 gene, DNA sequence, dairy cattle

INTRODUCTION

Brucella are Gram-negative facultative interacellular bacterial pathogens of humans and animals. The bacteria penetrate the mucosa of the nasal, oral, or pharyngeal cavities into hosts and are phagocytized by host macrophages, where survival and replication occurs. *Brucellosis* is a zoonosis that is difficult to control and charm off so that causes heavy economic losses and society problems. Human beings were infected in case, it was characterized with undulant fever and abortion, if untreated, can changed into a chronic infection with symptoms persisting for several months, chronic infections may result in infection of secondary tissues, including heart and brain (Baldwin and Goenka, 2006; Young, 1995). Symptoms may also recur years after the original infection. Pregnant cow is aborted and infertile owing to *B. abortus*. Cows affected with brucellosis badly foreclose the international trade of milk, milk products, beef and semen.

In China, Liu *et al*. (2005) isolated, identified, sequenced and characterized omp25 gene of *B. ovis* 80/019. Analysis of the nucleotide sequence revealed no absence presence in the omp25 gene from XinJiang Province of China (Liu *et al*., 2005), which differed from the sequence of *B. ovis* reference strain (accession number U33004) in which 36 bp near 3'-end of the omp25 gene was deleted to induce 12 amino acids deletion. It is indicated that the omp25 gene mutation can occurred in various regions of China, although, the studies showed the omp25 gene is highly conservative among *Brucella* sp. In this study, much of samples of milk of dairy cows were collected from the 9 Provinces (Gansu, Ningxia, Xinjiang, Shanxi, Shaanxi, Henlongjiang, Hainan, Shanghai and Inner Mongolia) in China and examined for brucellosis by nested-PCR (Cao *et al*., 2005; Qiu *et al*., 2005). The milk specimens from dairy cows confirmed positive for brucellosis with SAT and PCR were choose to be used in the test in order to search for mutation degree of the 25 genes of various field strains from different geographic regions by sequencing and phylogenetic tree analysis.

MATERIALS AND METHODS

Samples and total DNA preparation for PCR: Nine dairy cow milk specimens from Gansu, Shaanxi, Shanxi, Heilongjiang, Xingjiang, Ningxia, Shanghai, Hainan and

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Innermongolia Provinces which were confirmed positive for brucellosis with SAT and PCR were used in this study. Four hundred microliters sample were suspended in 100 μL 20% SDS and 100 μL TNE buffer (50 mM Tris-HC1, 500 mM NaC1, 125 mM EDTA, pH 8.0), the mixture samples were fully denatured by boiling for 10 min and then quenched on ice and digested for 4 h at 50°C by adding 10 μL of 20 mg mL^{-1} proteinase K and 20 μL of 50 mg mL^{-1} RNAase. DNA was purified twice with equal volume phenol-chloroform (25:24:1) and centrifuged at 8000×g for 10 min at 4°C, using 2.5 volumes of 100% ethanol precooled at -20°C and 1/10 volume of 5 M sodium acetate was added to precipitate the DNA for 2 h at -20°C. The recovered DNA was washed with 70% ethanol solution. The pellet was resuspended in 20 μL of nuclease-free water and stored at -20°C till used for DNA amplification.

PCR assay: In this study, the primer pairs used for nested-PCR amplification were designed on the basis of the published the omp25 gene of *B. abortus* (accession number X79284). First, the total DNA as template of the target gene was amplified by the primer: p1 (5'-AGCGCGGCATGGGCGGTTACT-3') and the reverse primer:p2(5'-GTTGCCTGTCCGGTTTTCCGTGTCC-3'), respectively. PCR amplifications were carried out using the 50 μL system and initiated by denaturing the sample for 5 min at 94°C, followed by 35 cycles at 94°C for 30 sec, 60°C for 1 min and 72°C for 1 min. After the last cycle the samples were incubated for an additional 10 min at 72°C before they were stored at 4°C. Second, the product above as template was amplified using the primer: p3 (5'-AACGTAATTTGTGTAAGGAGAATGC-3') and the reverse primer: p4 (5'-GTTTTCCGTGTCCAATTATGCT AT-3'), respectively, a fraction 694 bp in length was obtained including the whole omp25 gene ORF (Open Reading Frame) 642 bp in full length. The PCR reaction system were carried out by 30 cycle at 94°C for 30 sec, 58°C for 45 sec and 72°C for 1 min. After the last cycle samples were incubated for an additional 10 min at 72°C before they were stored at 4°C. The negative control was also set up in the PCR procedure. The amplified products were analyzed and affirmed by electrophoresis through 1% agarose gel with Ethidium Bromide (EB).

Sequence and data analysis: All the PCR-amplified products were purified by Agarose Gel DNA Purification Kit (TaKaRa) and cloned in pMD18-T vector (TaKaRa) by ligation enzyme adding 1 μL pMD18-T and 5 μL PCR products and the recombinant clone were screened using LB plate (50 μg mL ampicillin) adding X-gal and IPTG to inducing at 37°C overnight. The positive white colonies were chosen from the plate for multiplication. The recombinant plasmid was extracted and identified by PCR (the p3 and p4 primers) and digestion of *EcoRI* and *HindIII*. At last, the successfully recombined 9 clones were submitted to TaKaRa company for sequencing and then data analysis based on the published omp25 gene sequence of *B. abortus* (accession number X79284) using the DNAstar software. All the sequences were compared with the published omp25 gene sequence for detection of nucleotide diversity.

RESULTS

Nine omp25 genes of 9 *Brucella* field strains from brucellosis-affected dairy cows milk specimens were successfully characterized by PCR are shown in the Fig. 1 and all 9 PCR-amplified products cloned in pMD18-T vector were carried out for DNA sequencing. The results showed that every PCR-amplified product of the field strain omp25 gene was 694 bp in length in which a complete and 642 bp long ORF of omp25 was included and deduced a 212 amino-acid sequence.

Variability analysis of the nucleotide and amino acid sequences of the 9 *Brucella* field strains omp25 genes are shown in the Table 1. Variations in the nucleotides occurred at 17 positions in ORF of the omp25 analyzed, which caused 11 amino acids to mutate. A common character present in the 9 omp25 sequences, compared with the reference strain *B. abortus* were nucleotide substitutions CTC to CTG at the codon 118 and CGT to GGT at the codon 119 (mutation R119G) and the products of mutations were same as the other *Brucella* sp. Nucleotide substitutions TAT to TAC at the codon 55 were identified from the field strains in Gansu and Shaanxi Provinces and CTC to CTG at the codon 173 from the field strains in Gansu and Shanxi Provinces, but no amino acids mutations. Mutations TTC to TCC at the codon 16 (mutation F16S), GCT to GCC at the codon position 79 and TTT to CTT at the codon 114

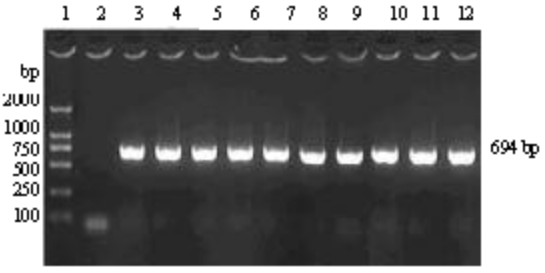

Fig. 1: The result of the nested-PCR, Lane 1: DL 2000 DNA Marker; Lane 2: The negative control, Lane 3-12: The PCR amplification products of the positive samples partly

Table 1: Nucleotide and amino acid differences in the omp25 gene of 9 Province

	Codon residue																
Origin	16 F TTC	50 G GGT	55 Y TAT	79 A GCT	114 F TTT	117 S TCG	118 T CTC	119 R CGT	134 T ACG	150 D GAC	160 G GGT	163 G GGT	171 N AAC	173 T CTC	178 Y TAC	179 R CGT	190 T CTG
B. abortus	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-
Gansu	-	-	TAC	-	-	-	CTG	GGT	-	-	GGC	-	AGC	CTG	-	-	-
Hainan	-	AGT	-	-	-	-	CTG	GGT	-	-	-	-	-	-	-	-	-
Heilongjiang	TCC	-	-	GCC	CTT	-	CTG	GGT	-	-	-	-	-	-	-	-	-
Inner Mongolia	-	-	-	-	-	-	CTG	GGT	TCG	-	-	-	-	-	-	-	-
Ningxia	-	-	-	-	-	-	CTG	GGT	-	GGC	-	GAT	-	-	-	-	-
Shanghai	-	-	-	-	-	-	CTG	GGT	-	-	-	-	-	-	CAC	-	-
Shaanxi	-	-	TAC	-	-	-	CTG	GGT	-	-	-	-	-	-	-	-	TTG
Shanxi	-	-	-	-	-	TCA	CTG	GGT	-	-	-	-	-	CTG	-	-	-
Xinjiang	-	-	-	-	-	-	CTG	GGT	-	-	-	-	-	-	-	CAT	-

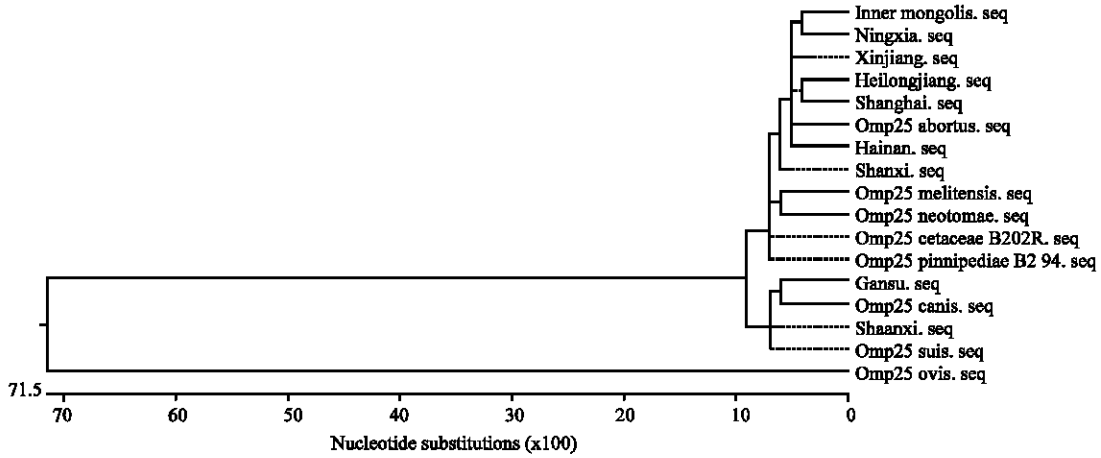

Fig. 3: The phylogenetic tree of the omp25 gene

(mutation F114T) was also identified in omp25 of *B. abortus* field strains from Heilongjiang Provinces. Nucleotide substitutions GGT to GGC at the codon 160 and AAC to AGC at the codon 171 (mutation N171S) were shown in the omp25 of *B. abortus* field strain from Gansu Province. Two mutations happened in omp25 of *B. abortus* field strain from Ningxia, namely the variations at codon position 150 (GAC to GGC with amino acid substitution D150G) and 163 (GGT to GAT with amino acid substitution G163D). In addition, five variations in nucleotides were detected at the codon position 50 (GGT to AGT with amino acid substitution G50S), the codon position 134 (ACG to TCG with amino acid substitution T134S), the codon position 178 (TAC to CAC with amino acid substitution Y178H), the codon position 179 (CGT to CAT with amino acid substitution R179H) and the codon position 190 (CTG to TTG with amino acid substitution T190L) in omp25 of *B. abortus* field strains from Hainan, Inner Mongolia, Shanxi, Shanghai, Xinjiang and Shaanxi Provinces, respectively.

The analytic result of the phylogenetic tress of omp25 gene of the 9 field strains from different Provinces was shown in the Fig. 2. The omp25 gene sequences of seven of the 9 field strains were identical with the reference strain of *B. abortus* but the omp25 sequences of the field strains from Gansu and Shaanxi Provinces were similar with *B. suis* (99.7 and 99.8%). The homology in all the sequences was higher than 98%.

DISCUSSION

Phylogenetically, the genus *Brucella* belongs to the *Rhizobizceae* group of bacteria (Yanagi and Yamasato, 1993), the DNA-DNA hybridization studies have revealed a high degree of homology (>90%) among the six recognized *Brucella* species (Verger *et al*., 1987, 2000) and isolated marine mammal strains (Cloeckaert *et al*., 2001, 2003) and the omp25 genes have high similarity (>98%) (Cloeckaert *et al*., 1995). In this research, analysis of the homology of the sequences further confirmed that the omp25 gene in *Brucella* species, biovars and strains were much conservative (98.2-100%). They were classied according to their apparent molecular mass as 25-27 and 31-34 kDa Omps, which belong to group 3 proteins and the seven genes were detected which were classified in four subgroups on the basis of percentage amino acid

sequence identities: Omp25 alone, the Omp25b-Omp25c-Omp25d cluster, the Omp31/31b subgroup and the less related Omp22 protein (also called Omp3b) (Salhi *et al*., 2003). Comparison and analysis of 9 omp25 sequences with the other gene of group 3 proteins shown that the homology was <40%.

The analysis of the sequences and phylogenetic tress of omp25 gene revealed the presence of differences in the omp25 gene of *Brucella* field strains in China 7 of 9 the omp25 sequences located at the same tree branch with the reference strain *B. abortus* (accession number X79284), except the sequences of the field strains from Gansu and Shaanxi Provinces, which were much more near to the *B. suis* (accession number U39397). Dairy cows were affected with *B. suis*, which has been reported in China. The field strains from Xingjiang, Ningxia and Inner Mongolia Provinces had very high homology of omp25 genes because of their borders being connected each other in the Northwest of China. But, the sequences of omp25 genes of the field strains from Heilongjiang and Shanghai have a very close relationship, while distance of the 2 Provinces is very far; the same coincidence also occurred between Hainan and Shanxi Provinces. The result above demonstrated that the omp25 gene identities of the *Brucella* field strains from the mainland appeared to be not related the geographic places where the field strains collected from. Most studies focused on the characterization of omp25 have revealed that the omp25 gene is a virulence factor (Edmonds *et al*., 2001; Jubier-Maurin *et al*., 2001) and the *Brucella* omp25 mutant was shown to be attenuated in host (Edmonds *et al*., 2002a, b). However, slight mutation of *B. abortus* omp25 of the field strains in China appeared to cannot reduce their virulences for hosts and *B. abortus* caused pregnant cows abortions were very serious in the various areas in China.

Comparing the sequences of the omp25 genes of *Brucella* field strains from the 9 Provinces in China with other *Brucella* sp. including the *B. pinnipediae* B294 and *B. cetaceae* B202R. The mutations had occurred at the codon position 55 of the omp25 of the field strains from Gansu and Shaanxi Provinces, which caused mutation GGT to GGC, being identical with the other *Brucella* sp. Obviously, the characterization of sequence at the codon position 173 (CTC to CTG) in field strains from Gansu and Shaanxi Provinces showed that they were very close to *B. suis* (accession number U39397). In addition, variations at the codon position150 (GAC to GGC) induced the amino acid substitution D to G, being identical with the omp25 of *B. canis* (accession number U39358). The phylogenetic tree of omp25 gene has revealed the sequences of the field strains from Gansu and Shaanxi Provinces were closed to the *B. suis* (99.7 and 99.8%). Therefore, it is presumed that cows affected with brucellosis may come from *B. suis* prevalence regions in China. This presumption will can be approved by further experiments of isolation and identification of the *Brucella* sp. from animals with brucellosis.

CONCLUSION

In this study, it is indicated that the omp25 gene mutation can occurred in various regions of China, although the studies showed the omp25 gene is highly conservative among *Brucella* sp. The analysis of the sequences and phylogenetic tress of omp25 gene revealed it is presumed that dairy cows were affected with *B. suis*, which has been reported in China. These data is valuable to study the evolution and variation of *Brucella* in China.

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