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Short Communication

Brucella suis S2 Isolated from Aborted Sheep Fetuses in Northwestern China

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Abstract

This study aimed to investigate the cause of abortion in a traditional sheep farm. Here, 31 specimens were examined by PCR targeting the *Brucella* outer membrane protein gene 22 *(omp22),* and 25 (80.6%, 25/31) were found to be positive. Totally, 3 *Brucella suis* S2 and 10 *Brucella melitensis* were isolated from 31 aborted fetuses in which one *B. suis* S2 and *B. melitensis* were isolated from a same aborted fetus. All of these isolates were identified by PCR and conventional bacterial tests. These 3 *B. suis* isolates and the reference strain *B. suis* S2 were all identified as *B. suis* biovar 1 and the 10 *B. melitensis* isolates were all identified as *B. melitensis* biovar 3. This study suggests that *B. suis* S2 can partially induce abortion in pregnant ewes. Hence, for the sake of safety, it is need to develop a new *Brucella* vaccine to protect pregnant sheep from brucellosis.

Keywords: *Brucella suis S2, Sheep, Abortion, China*

Kuzeybatı Çin'de Atık Koyun Fötuslarından *Brucella suis* S2 İzolasyonu

Öz

Bu çalışma, geleneksel bir koyun çiftliğinde atıkların sebebini araştırmak amacıyla gerçekleştirilmiştir. Çalışmada, 31 hayvan PCR tekniği ile *Brucella* dış zar protein geni 22 *(omp22)* için taranmış ve 25'i (%80.6, 25/31) pozitif bulunmuştur. Otuz bir adet atık fötusun 3'ünden *Brucella suis* S2 ve 10'undan *Brucella melitensis* izole edilirken bir hayvanda *B. suis* S2 ve *B. melitensis* beraber tespit edilmiştir. Tüm bu izolasyonlar hem PCR hem de klasik bakteriyolojik testlerle yapılmıştır. Üç adet izole edilen *B. suis* ve referans suş *B. suis* S2 B. suis biovar 1 olarak, 10 adet izole edilen *B. melitensis* ise *B. melitensis* biovar 3 olarak identifiye edildi. Bu çalışma, *B. suis* S2'nin gebe koyunlarda atığa sebep olabileceğini göstermiştir. Bu nedenle, gebe koyunlarda koruyucu amaçlı brusellozise karşı yeni bir aşı geliştirmeye gereksinim olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Brucella suis S2, Koyun, Atık, Çin*

INTRODUCTION

The brucellosis causes great losses among domestic animals throughout the world, and it has been prevalent for decades in China [1]. Long-term serological studies have indicated that 5% of sheep and 0.8% of cattle are infected with brucellosis. *Brucella* melitensis *(B. melitensis)* infection is endemic, particularly in developing countries in the Mediterranean and Middle East and parts of Africa and Latin America [2]. It is also the main cause of sheep abortion in China. The seropositive rate was 0.66% for cows and 3.25% for sheep during the two-year period covering 2013-2014 in China [3]. In areas where the brucellosis morbidity of sheep and goats is high, vaccination is the best method of controlling the disease in animals [4].

In China, an attenuated strain of *Brucella* suis *(B. suis)* S2 was obtained by serial transfer of a virulent *B. suis* biovar 1 strain originating from swine [5]. It is widely used in sheep and goats and is administered orally in their drinking water.

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Although it has been assessed for safety under field conditions and found satisfactory by Chinese authorities, there remain a few controversial issues regarding specific risks. It has been reported that the infection and replication of *B. suis* S2 in goat trophoblast cells (GTCs) can induce apoptosis due to endoplasmic reticulum (ER) stress, which is involved in the mechanism underlying goat abortions [6]. Verger et al.[5]. have also reported that *B. suis* S2 induces a poor immunity against the *B. melitensis* infection of pregnant ewes.

This study was performed on a traditional farm in the Ili region, where located in the northwest of Xinjiang Uygur Autonomous Region (XUAR) in China. Previous works have observed that the rate of abortion is relatively high on a neighboring farm where brucellosis is epidemic. There were about 157 ewes on this farm and all of the animals were given adequate food and water. The purpose of the present work was to investigate the reason for these abortions on this farm.

MATERIAL and METHODS

Ethical Approval

All animals used in our experiment were treated humanely and in accordance with institutional animal care guidelines. Our study was approved by the Animal Care and Use Committee of Shihezi University.

Bacterial Strains

Reference strains of *B. melitensis* 16M and *B. suis* S2 were provided by the Anthropozoonosis laboratory in Shihezi University, *B. suis* 1330 was donated by the College of Veterinary Medicine, Northwest A&F University.

Sample Collection and DNA Extraction

The samples including sheep aborted fetuses (n=31) were collected from the Ili region northwest of Xuar in areas in which brucellosis is common between April and May in 2018. Samples of spleen, liver, and lung tissues were collected aseptically from aborted sheep fetuses. Then the tissue samples were cut into pieces weighing about 6 mg, suspended in 1 mL sterile saline, homogenized using a tissue grinder for 15 min, and centrifuged at 10.000 rpm for 1 min. The supernatants were discarded. DNA extraction procedures were performed using the TIANamp Genomic DNA Kit (Tiangen Biotech Co., Ltd., China) according to the manufacturer's instructions. DNA concentrations were determined by measuring the A_{260}, and the samples were stored at -20°C until further processing.

PCR Amplification

The forward (F) and reverse (R) primers of *omp22* gene were F 5'-TGATGGGAGGGACCGACTA-3' and R 5'- TGGTTC TTCAGGTTGTTACGC-3', which were used to screen *Brucella* spp. The Bruce-ladder multiplex PCR primers were used to identify the species of *Brucella* genus [7]. The duplex PCR primers were used to differentiate *B. suis* S2 from *B. suis* 1330 [8]. All samples were examined by PCR in a total volume of 30 µL, with 13 µL ddH_2O, 15 µL master mix, 0.5 µL of each primer and 1.5 µL DNA template. The reaction mixtures for *omp22* were first incubated for 5 min at 94°C. Then 37 cycles were performed as follows: 30 s at 94°C, 40 s at 55°C, and 5 min at 72°C. The reaction was performed in a DNA thermal cycler (Perkin-Elmer, USA) and 2 µL of the product was, fractionated in a 1.5% or 2% agarose gel, stained with 0.5 mg/mL ethidium bromide solution, and visualized under UV light [9]. All of these data were analyzed using SPSS version 17.0.

Bacterial Isolation

The tissue samples were homogenized before plating on the Brucella-selective agar (BD, USA). Then, 100 µL of the homogenized or lysed suspension was inoculated onto two Brucella-selective agar plates (BD, USA). The suspension was spread with a loop producing a depot followed by single colonies. All cultures were incubated at 37°C with 5% CO_2 for five days. *Brucella* identification and species differentiation were accomplished using PCR protocols [7]. *Brucella* was identified by conventional bacterial and typing methods [10]. This process was completed at the Center for Disease Prevention and Control (CDC) of China.

RESULTS

Of the 31 studies samples, PCR results targeting the *omp22* gene amplification demonstrated that 25 of the aborted sheep fetuses were found positive with *Brucella* spp. Among the positive results, 2 out of 25 aborted fetuses were identified as *B. suis* by Bruce-ladder multiplex PCR. PCR using DNA from *B. melitensis* amplified six fragments, of 1682, 1071, 794, 587, 450 and 152 bp in size; with *B. suis* by the presence of an additional 272-bp fragment *(Fig. 1)*. These isolates were further identified as *B. suis* S2 using duplex PCR. The *B. suis* 1330 was positive in the duplex PCR for the 285 bp amplicon. The vaccine strain *B. suis* S2 was positive in the duplex PCR for both the 285 bp and 497 bp amplicons *(Fig. 2)*. The remaining 23 samples were infected with *B. melitensis*, only partial results were presented *(Fig. 1)*.

Totally, *Brucella* was isolated from 13 out of 25 samples and the isolates were confirmed using the *omp22* gene-targeting PCR (data not shown). *B. suis* S2 was isolated from 2 out of 25 positive samples and identified using duplex PCR. *B. melitensis* was isolated from 9 out of 25 positive samples. Apart from these, *B. melitensis* and *B. suis* S2 were simultaneously isolated from one aborted fetus *(Table 1)*. Thus, totally 3 *B. suis* S2 and 10 *B. melitensis* were identified from aborted fetus. Three wild isolates and the reference strain of *B. suis* S2 were all identified as *B. suis* biovar 1 by conventional bacteriological methods. All of these three wild isolates and *B. suis* S2 were found positive for

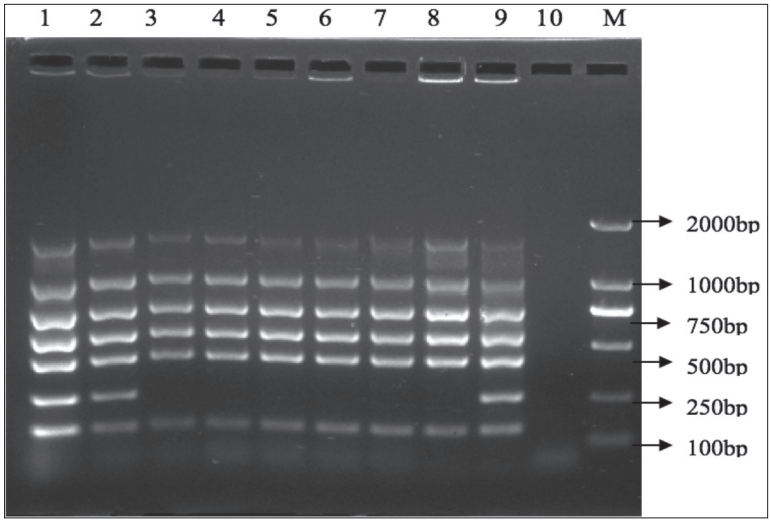

Fig 1. Bruce-ladder multiplex PCR: differentiation of *Brucella*, *B. melitensis* and *B. suis*

Lane 1-7: aborted sheep fetuses; Lane 8: *B. melitensis* 16M Lane 9: *B. suis* S2; Lane 10: Negative Control; Lane M: DL2000 (Takara)

Fig 2. PCR product for differentiation of vaccine strain *B. suis* S2 from *Brucella* wild strain 1330.

Lane 1-2: aborted sheep fetuses 1; Lane 3-4: aborted sheep fetuses 2; Lane 5: *B. suis* 1330; Lane 6: *B. suis* S2; Lane 7: Negative Control; Lane M: DM500 (Takara)

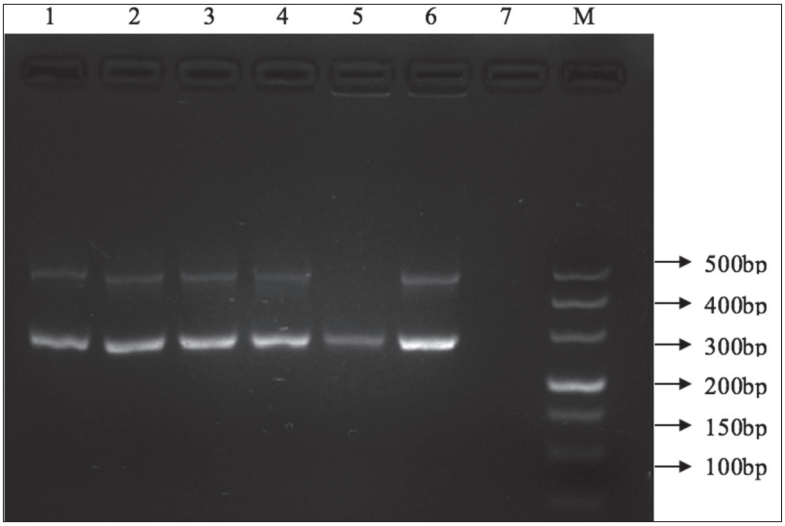

Table 1. *The isolation of B. suis S2 and B. melitensis in aborted fetuses*

Animal No	PCR Results	*B. melitensis*	*B. suis* S2
1	+	-	+
2	+	-	+
3-11	+	+	-
12	+	+	+
13-25	+	-	-
26-31	-	-	-

H_2S production, thionine sensitivity, tbilisi phage lysis RTD 10^4 and A monospecific sera agglutination. The growth of all the *B. melitensis* isolates on medium with thionin at only 40 µg/mL (1:25.000) concentration and basic fuchsin at all concentrations suggested these isolates as *B. melitensis* biovar 3.

DISCUSSION

Various pathogens, such as *Coxiella burnetii*, *Chlamydophila abortus*, *Salmonella enterica* serovar Abortusovis, *Toxoplasma gondii*, and *Neospora caninum*, have been found to induce abortions in pregnant sheep [11], but in this study, we could not find any of these pathogens in the samples after PCR identification and pathogen isolation (data not shown). Here, 25 (80.6%) out of 31 samples were identified as being infected with *Brucella* by the *omp22* target gene PCR assay. However, three isolates were further identified as *B. suis* S2, and the rest of 10 isolates were identified as *B. melitensis* according to the Bruce-ladder multiplex PCR assay [7]. These results suggest that *B. suis* S2 could partially induce abortion in pregnant ewes because *B. suis* S2 can infect and replicate in GTCs; the growth rates begin to accelerate at 12 h, with the bacterial load peaking after 24 h [6]. In addition, it is likely that the particularly high sensitivity of pregnant animals to brucellosis involves the local suppression of the immune response in the placenta [12], which leads to colonization and placenta damage, and ultimately abortion, in pregnant animals.

The bacteriological isolation is still the "gold standard" for diagnosis of Brucellosis [2]. The rate of isolation of *B. melitensis* from aborted cattle and sheep fetuses were found to be

28% in Ili region (northwest of XUAR) [13]. In this study, 10 *B. melitensis* (32.2%) and 3 *B. suis* S2 (9.6%) were isolated from 31 aborted sheep fetus samples in which one *B. suis* S2 and *B. melitensis* were isolated from a same aborted fetus *Table 1*. The co-existence of two different species of *Brucella* in the same animal is rare, and the mechanism by which they co-exist is not fully understood. However, there are still 13 samples positive for PCR but negative for culture because of the contamination decreased the rate of *Brucella* isolation. Thus, the techniques of *Brucella* isolation need to be improve in our laboratory. The Brucellosis is still enzootic in Ili region of XUAR and is the main cause of abortion in sheep and cattle. *B. melitensis* and *B. suis* strains are biotyped by 4 main tests such as H_2S production, CO_2 requirement, dye (thionin and basic fuchsin) sensitivity and agglutination with monospecific A and M antiserum [2]. *B. melitensis* biotype 3 is the predominant subtype of *B. melitensis* as documented before [13]. In parallel with this, current study revealed that 76.9% (10/13) isolates belonged to *B. melitensis* biotype 3 while *B. suis* biovar 1 made up 23.0% (3/13).{Alton, 1988 #168}

The live *B. suis* S2 vaccine has been used successfully in China to immunize sheep for decades. However, the occurrence of abortion in ewes after vaccination suggests that several factors may play a role in vaccine-induced abortions, including immune status, current health status, other diseases, and vaccines [14]. The present work shows that the vaccine strain could induce the abortion and be isolated from aborted sheep fetus. Hence, it is urgently necessary to develop a new *Brucella* vaccine to protect animals especially pregnant animals more safely.

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