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Original Article

A Case of Identity Confirmation of *Brucella abortus* S99 by Phage Typing and PCR Methods

Alamian [1, *], S., Dadar [1], M., Solimani [2], S., Behrozikhah [1], A.M., Etemadi[1], A.

1. Department of Brucellosis, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Karaj (AREEO), Iran

2. Department of Bio bank, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

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Corresponding Author: alamiansaeed@gmail.com

ABSTRACT

Brucellosis is a zoonotic infection that is associated with fever in humans and abortion in animals. The agent of this disease is a facultative intracellular gram-negative coccobacillus called *Brucella*. There are six classic species, including *B. abortus, B. melitensis, B. suis, B. canis, B. neotomae*, and *B. ovis*. In recent years, four new species have been reported, including *Brucella ceti, B. microti, B. pinnipedialis, and B. inopinata*. Human disease causes hygienic and economic losses, including inactivity of workforces in the community and high cost of treatment. The disease also causes catastrophic losses in the livestock industry. There is no effective vaccine against human brucellosis. Hence, attempts to prevent human infection with *Brucella* are focused on preventative measures, including control of infection in livestock, which lead to a reduction in its incidence in humans. The common methods for diagnosis of this disease are serologic methods including Rose Bengal, Wright -2 ME and the ring test. *B. abortus* strain S99 is used to produce these diagnostic antigens. The production of these antigens requires the presence of a well-characterized seed with full identity. The aim of this work was confirmation of the identity of *B. abortus* S99 by phage typing, AMOS and multiplex PCR techniques. Therefore, it is essential to carry out the identification of the strains used as seed for the production of the brucellosis diagnostic antigens. In this project, *B. abortus* strain 99 was supplied by the bacterial collection of the Brucellosis Department of Razi Vaccine and Serum Research Institute. Then, the main aim of the present study was the confirmation of the seed identity by doing the tests through the standard phage typing method, AMOS PCR and multiplex PCR (Brucladder) methods. Results were in support of the identity of the studied strain, and the molecular methods could also be used as the sensitive approaches for validation of antigenic seed.

Keywords: Antigenic seed, *Brucella abortus* S99, Molecular test, Validation

Un cas de Confirmation d'Identité de *Brucella abortus* S99 par Typage du Phage et Méthodes PCR

Résumé: La brucellose est une zoonose à l'origine de fièvre chez l'homme et d'avortement chez l'animal. L'agent de cette maladie est un coccobacille Gram-négatif intracellulaire facultatif appelé *Brucella*. Il existe six espèces principales, à savoir *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. neotomae* et *B. ovis*. Ces dernières années, quatre nouvelles espèces ont été signalées, à savoir *Brucella ceti*, *B. microti*, *B. pinnipedialis et B. inopinata*. Chez l'homme, cette maladie est souvent débilitante entraînant des pertes économiques, considérables. La maladie provoque également des pertes catastrophiques dans le secteur de l'élevage. Il n'y a pas de vaccin efficace contre la brucellose humaine. Par conséquent, les tentatives de prévention des infections zoonotiques sont axées sur des mesures préventives dans le but de contrôler l'infection chez le bétail afin de

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réduire son incidence chez l'homme. Les méthodes habituelles de diagnostic de cette maladie sont les méthodes sérologiques, notamment l'agglutination de Rose Bengal, Wright -2 ME et le ring-test. La souche S99 de *B. abortus* est utilisée pour produire les antigènes dédiés au diagnostic. La production de ces antigènes nécessite la présence d'une souche bien caractérisée et pleinement identitaire. Le but de ce travail était de confirmer l'identitéde *B. abortus S99* par typage sur phage, par les techniques de PCR AMOS et Multiplex. Par conséquent, il est essentiel de procéder à l'identification des souches utilisées pour la production des antigènes utilisés dans le diagnostiques de la brucellose. La souche 99 de *B. abortus* étudiée dans ce projet provenait de la collection bactérienne du département de brucellose de l'Institut de recherche sur le vaccin et le sérum de Razi. L'objectif principal était ensuite de confirmer l'identité de cette souche en effectuant une batterie de tests parlysotypage, PCR AMOS et PCR multiplex (Brucladder). Les résultats ont confirmé l'identité de la souche étudiée et les méthodes moléculaires présentées dans cet article constituent une approche sensible pour la validation des souches dédiées à la production d'antigénes.

Mots-clés: Test moléculaire, Souche antigénique, *Brucella abortus*

INTRODUCTION

Brucellosis is known as bacterial zoonosis with worldwide distribution induced by facultative intracellular, non-spore-forming, Gram-negative bacteria of genus *Brucella* that affecting animals and humans (Bricker and Halling, 1994; Wareth et al., 2017). It is a very critical public health issue in developing countries ((FAO), 2010) that affects different body organs and induces periods of chronicity, reinfection, and relapse (Young, 1995). The causative agent of bovine brucellosis is known as *Brucella abortus*, which causes economic and industrial loss, because of reduced milk yield, spontaneous abortion, and limitation of animal movement in cattle (Nielsen, 2002; Júnior et al., 2017). In livestock, the transmission of *B. abortus* is commonly induced by direct contact to vaginal discharges or tissues and fluids from infected fetuses after abortion. Also, inhalation, ingestion of contaminated pasture or water with fetal tissues and fluids, sexual contact, intermediate reservoirs, and artificial insemination with contaminated semen could be considered in *Brucella* transmission (Wareth et al., 2017). Currently, *Brucella* agglutination tests have been done to measure both immunoglobulin M (IgM) and IgG *Brucella* antibody titers (Nielsen, 2002; Júnior et al., 2017). In addition, *Brucella* agglutination tests such as standard tube agglutination (STAT Wright) and slide agglutination (Rose-Bengal) tests have been applied to serum samples of suspected samples to track the brucellosis patients and infected animals (Mert et al., 2003). For this purpose, the *B. abortus* S99 that contains several antigenic components has been used to determine the immune response and *Brucella* pathogenesis and appeared to be remarkably useful in *Brucella* diagnosis (Araj, 2010). According to standard procedures, *B. abortus* strain S99 is considered an antigenic strain for producing brucellosis diagnostic antigens in reference laboratories. The antigens of *B. abortus* S.99 are used in numerous countries for the diagnosis of this disease and have been accepted as favorable assay because it is an easily performed, reliable, and cheap test that would help in differentiating these disorders (Cunningham et al., 1980; Goktas et al., 1991). Numerous researcher and studies have used the official S-type smooth *B. abortus* strain 99 as the best antigenic source for different serological tests of *Brucella* diagnosis (Cunningham et al., 1980; Goktas et al., 1991; Adone et al., 2008). The biosafety and quality controls in the antigenic source must be put in place to inhibit any contamination of

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antigenic seed and prevent cross-reaction with other bacteria that have low specificity due to serologically cross-reacting bacteria (Bounaadja et al., 2009). For this reason, a seed lot system has been established as a system for successive batches of a product which are derived from the seed in serological tests. The approved seed lot system should meet all requirements of WHO regulations for different isolates of *B. abortus* S99. On the other hand, the S99 antigen strain applied should be recognized by historical records that include information of subsequent manipulation and its origin. It would also be recommended for the master seed lot to have identified proven studies on a batch derived from it by a production process that is representative of the commercial process. Therefore, this study aimed to improve the validation of the seed lot system for antigen products of *B. abortus* S99.

MATERIAL AND METHODS

Sample collection. The sample examined included the *B. abortus* S99 supplied by the Veterinary Laboratories Agency (VLA) of Weybridge, United Kingdom, which was stored in the biobank of Razi Vaccine and Serum Research Institute, Brucellosis Vaccine and Antigens Production Department during the time of the study (Karaj, Iran).

Bacterial identification and biotyping. Bacteriological analysis was performed under suitable protection in safety hoods at the Brucellosis Vaccine and Antigens Production Department. Classical biotyping was done according to Alton et al. (Alton et al., 1988b). Monospecific antisera of *Brucella* including A and M as well as *Brucella* reference Tbilisi (Tb) and Izatnagar (IZ) phages were routinely prepared and applied for identification and analysis in this lab. A panel of biotyping tests, including H_2S production, CO_2 dependence, growth in media containing thionin and basic fuchsin, agglutination with specific *Brucella* A and M antisera, agglutination by acriflavine, and lysis by specific phages were done (Godfroid et al., 2013) and the results were interpreted according to the OIE manual (http://www.oie.int/en/animal-health-in-the-world/ animal-diseases/brucellosis/).

Molecular typing. Crude genomic DNA of *B. abortus* strain 99 was extracted using high pure PCR template preparation kit (Roche, Germany) according to the manufacture's protocol. Briefly, 200 µl of PBS was added to the bacteria pellet and mixed with 200 µl binding buffer and 40 µl proteinase K. Afterward, it was mixed immediately and incubated at 70 °C for 10 min. Then, 100 µl isopropanol was added and mixed well and finally, a filter tube was used for washing and elution of DNA. The integrity of DNA was checked using 2% agarose gel. Also the DNA concentration was examined by reading at 260/280 nm using the Nanodrop Spectrophotometer (Wilmington, DE, USA) and stored at – 20 °C until further analysis (Table 1) (Song et al., 2012). The extracted DNA was subjected to IS*711*-based PCR for the *Brucella* spp. under the following conditions: Step 1: 95 °C 5 min, Step 2: 95 °C 30 s, Step 3: 55 °C 60 s, Step 4: 72 °C 3 min, and Step 5: 72 °C 10 min. Steps 2, 3, and 4 were repeated in 35 cycles (Ewalt and Bricker, 2000). Species-level molecular identification was also performed using multiplex PCR (Bruce-ladder) under the following conditions: Step 1: 95 °C 5 min, Step 2: 95 °C 30 s, Step 3: 56 °C 90 s, Step 4: 72 °C 3 min, and Step 5: 72 °C 10 min. Steps 2, 3, and 4 were repeated in 30 cycles (López-Goñi et al., 2008). The amplified products were resolved by electrophoresis using a 2 % agarose gel. All used primers are mentioned in Table 1.

RESULTS

Bacteriological examination confirmed *B. abortus* strain 99 as *B. abortus* biovar 1, and was preserved in the biobank of Razi Vaccine and Serum Research Institute, Brucellosis Vaccine and Antigens Production Department. Common phenotypic features that are typical for *Brucella* spp. have been approved from *B. abortus* S99 seed. The isolate grew in 10% carbon dioxide (CO2) after three days incubation at 37 °C. Furthermore, the *B. abortus* S99 seed was Gram-

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negative coccobacilli (Figure 1) and produced small honey colored, translucent and shiny colonies with a smooth surface (Figure 2). *B. abortus* S99 seed was characterized as biovar 1 at the species level for the seed sample by using Bruce-ladder multiplex PCR and AMOS PCR. The isolate was confirmed as *B. abortus* in the Bruce-ladder PCR with PCR products of 1682, 794, 587, 450, and 152 bp in size (Figure 3). As expected, the 498 bp *B. abortus* specific band was seen in AMOS PCR, which detects only biovars 1, 2, and 4 *B. abortus* (Figure 4).

DISCUSSION

The rate of human brucellosis is not exactly known in developing countries and it remains a health problem ((WHO), 2011), although it is a controlled disease in developed countries (Mert et al., 2003). Several commercial and in-house serological tests have been applied for investigating patients with brucellosis (Ruiz-Mesa et al., 2005; Gomez et al., 2008). The serological tests such as indirect Coombs test, wright test, 2ME test, enzyme-linked immunosorbent assay (ELISA), indirect enzyme-linked immunosorbent assay (iELISA), milk ring test (MRT), and Rose Bengal test (RBT) are currently applied in the brucellosis diagnosis as very specific and sensitive assays. In addition, by the application of agglutination tests, brucellosis can be effectively excluded from the diseases with similar clinical features (Reddy et al., 2014; Getachew et al., 2016; Ahasan et al., 2017). On the other hands, the standardization of reference antigens is critical for validation of the diagnostic kit and antigens and could influence the test results. Moreover, the detection of antibodies of *Brucella* infections could be done by commonly used diagnostic tests such as Rose Bengal slide agglutination, serum agglutination test (SAT), and Indirect Coombs (antihuman globulin) test through the standard whole *B. abortus* cells as antigens (Nielsen, 2002; Araj, 2010). According to national and international standards such as GMP, the use of bacterial strains for the production of biological products such as diagnostic kits should be under seed-lot system requirements. The system is mainly based on a two-tire assembly consisting of master and working seed ((WHO), 2011; Manual, 2017). The *B. abortus* S99 product is produced from the working seed, while a working seed is processed from the master seed in a routine production system without more passages from

Table 1. Primer sets and expected amplicon sizes specific for the different Brucella species

Strain amplicon	Primer set	Primer sequence (5-3’)	DNA target	Size (bp)	References
B. abortus	IS711 AB	TGCCGATCACTTTCAAGGGCCTTCAT GACGAACGGAATTTTTCCAATCCC	IS711	498	(Ewalt and Bricker, 2000)
B. melitensis	IS711 BM	TGCCGATCACTTTCAAGGGCCTTCAT AAATCGCGTCCTTGCTGGTCTGA	IS711	731	(Ewalt and Bricker, 2000)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI0998f BMEI0997r	ATC CTA TTG CCC CGATAA GG GCT TCG CAT TTT CACTGT AGC	Glycosyltransferase, gene wboA	1,682	(López-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI0535f BMEI0536r	GCG CAT TCT TCG GTTATG AA CGC AGG CGA AAA CAGCTA TAA	Immunodominant antigen, gene bp26	450	(López-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI1436f BMEI1435r	ACG CAG ACG ACC TTCGGTAT TTT ATC CAT CGC CCTGTCAC	Polysaccharide deacetylase	794	(López-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEII0428f BMEII0428r	GCC GCT ATT ATG TGGACT GG AAT GAC TTC ACG GTCGTT CG	Erythritol catabolism, gene eryC (Derythrulose-1-phosphate dehydrogenase)	587	(López-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEII0987f BMEII0987r	CGC AGA CAG TGA CCATCA AA GTA TTC AGC CCC CGTTAC CT	Transcriptional regulator, CRP family	152	(López-Goñi et al., 2008)

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the master seed to be satisfactory regarding safety and efficacy.

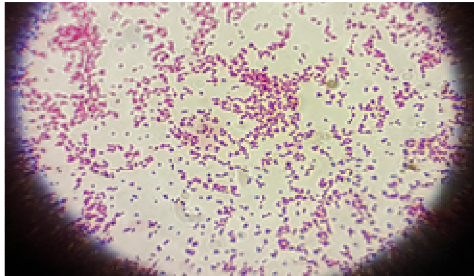

Figure 1. Gram-stained smear of *B. abortus* strain 99 in *Brucella* agar after 72 hr. (1000x magnification).

Figure 2. Smooth, honey-colored colonies of B. *abortus* strain 99 grown in *Brucella* agar after 72 hr.

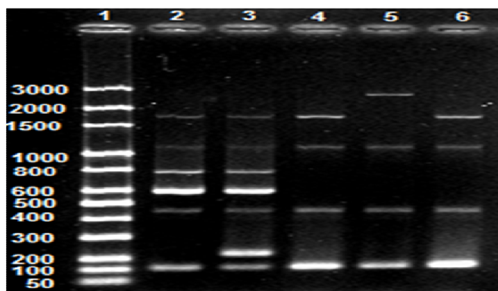

Figure 3. Ethidium bromide-stained agarose gel electrophoresis of multiplex PCR (Brucladder). The bands in lane1 show DNA ladder 100, lane 2 *B. melitensis* 16M, lane 3 *B. melitensis* Rev1, lane 4 *B. abortus* 544, lane5 *B. abortus* RB51, and lane 6 *B. abortus* S99.

In addition, unwanted changes that might arise from repeated subcultures or multiple generations of *B. abortus* S99 could be controlled by the passage history and the origin of the working antigenic seed and the master antigenic seed. Therefore, the production of antigenic seed products obtained by microbial culture should be performed according to a system of master and working seed lots and/or cell banks. Documented detailed identification and full information for all bacterial strains are also essential elements of a fully functional seed lot system.

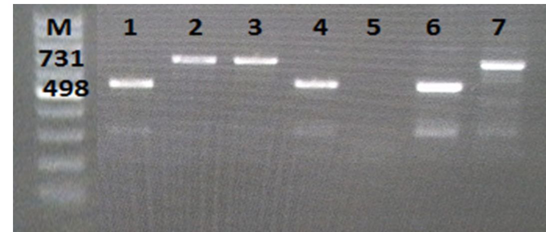

Figure 4. Multiplex PCR (AMOS PCR). The bands in laneM show DNA ladder 100, lane 1 B. abortus 544, lane 2 B. melitensis 16M, 3 B. melitensis Rev1, lane 4, B. abortus RB51, lane5 Control negative, lane 6 B. abortus S99, and lane 7 B. melitensis biovar 1.

The identification of microorganisms consists of a series of different biochemical, serological, and molecular tests and techniques (Alton et al., 1988a). On the other hand, Iran is an endemic country of brucellosis and serodiagnosis of this zoonosis is hotly demanded (Zowghi et al., 2008; Godfroid et al., 2013). Therefore, the elaboration of a good screening test for identification of brucellosis should be done according to a confirmed antigenic master and working seed with a high sensitivity serologic test (Hanci et al., 2017). In addition, with the help of a good antigen *Brucella* seed, the serological test evaluation is objective and simple (Manual, 2017). Besides, the identification of *Brucella* antibodies by rapid and accurate titration also exhibits other critical advantages. According to the results of this study, *B. abortus* S99 (biovar 1) that was obtained from the seed antigenic collection of Razi Institute of Iran could be routinely grown on *Brucella* agar medium (Himedia, India) at 37±1 °C for 72 hr. In addition, the molecular and biotyping of *B. abortus* S99 would be recommended as specific approaches to identify the quality of the antigenic seed in antigen products. The *B. abortus* S99 showed a promising feature; even among different seeds stored during different years. Furthermore, the results of this method appeared to be particularly useful provided that the biotyping and molecular tests of the master seed and the working seed are well-determined.

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Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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