



EXTRACTION AND CHARACTERISATION OF *BRUCELLA ABORTUS* STRAIN RB51 ROUGH LIPOPOLYSACCHARIDE

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Summary

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Brucellosis is an important zoonotic disease with considerable impacts on human and animal health. *Brucella abortus* strain RB51 vaccine is used for prevention of bovine brucellosis in Iran. Due to strain roughness, available serological tests cannot detect vaccinated animals. Detection of serological responses to the vaccine is important to monitor accurate vaccination implementation. Rough lipopolysaccharide (RLPS) of RB51 strain was extracted and characterised to develop serological tests for diagnosis of vaccinated animals. RLPS was extracted using phenol-chloroform-petroleum ether and evaluated by limulus amoebocyte lysate (LAL) assay, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and agar gel immunodiffusion (AGID). According to our results, the extracted RLPS caused positive reaction in LAL assay. In SDS-PAGE, a band with a molecular weight around 14 kDa was identified after specific staining using silver nitrate. Double AGID of the RLPS with a hyperimmune serum resulted in a precipitation line formation. Our study showed that the method can be successfully used to extract RLPS from *Brucella abortus* strain RB51 as confirmed by LAL assay, PAGE and AGID.

Key words: brucellosis, RB51 vaccine, rough lipopolysaccharide

INTRODUCTION

Brucellosis is an important bacterial zoonosis which remains a human and animal health problem in many countries (Godfroid *et al.*, 2011). It is caused by Gram-negative coccobacilli belonging to the genus *Brucella* which are aerobic, non-motile and facultatively intracellular parasites (Seleem *et al.*, 2010). Each

Brucella species has a preferred animal host (Poester *et al.*, 2013). Bovine brucellosis is mainly caused by *Brucella abortus* while other species such as *B. melitensis* and *B. suis* have been also implicated. In cattle, the disease is characterised by reproductive problems including abortion, stillbirth, infertility and decline in milk

production imposing considerable economic losses. It can also pose public health hazards through direct and indirect contact with infected animals and contaminated animal products (Olsen & Tatum, 2010). Vaccination is an effective strategy to control the disease in susceptible animal hosts for which live attenuated vaccines are widely used (Olsen & Stoffregen, 2005). *Brucella abortus* strain 19 and strain RB51 are two vaccines recommended by World Organization for Animal Health (OIE) for active immunisation of cows (OIE, 2012a). Vaccination induces cell mediated immune responses (CMI) conferring protection against abortions and reducing bacterial shedding from infected animals. Hence, it can decrease exposure to the pathogenic bacteria and brucellosis prevalence in cattle herds (Olsen & Stoffregen, 2005; Olsen & Tatum, 2010).

RB51 vaccine strain was introduced in 1990s. It was developed by successive passages of a virulent parent strain designated as 2308, on culture media containing rifampicin and penicillin antibiotics. It was proved that RB51 strain lacks perosamine O-side chain of the LPS responsible for smooth phenotype in *Brucellae* (Schurig *et al.*, 1991). The rough morphology of the strain provides an advantage when used as a vaccine against bovine brucellosis. It does not provoke production of antibodies against smooth LPS which are detected in serological tests used for identification of infected animals (Moriyon *et al.*, 2004). This is considered as a limitation of vaccination with smooth S19 vaccine (Olsen & Tatum, 2010). Therefore, since there is no interference with serodiagnosis of the disease, concomitant implementation of vaccination and test-and-slaughter is possible. Although there are some debates about S19

vaccine superiority in efficacy (Olsen & Stoffregen, 2005; Olsen & Tatum, 2010), it has been replaced by RB51 for the vaccination of calves and cows in many countries including Iran due to this advantageous feature.

However, there is no serological test to detect antibodies induced by RB51 strain following vaccination. Identification of post-vaccination serological responses in vaccinated animals is important to monitor accurate implementation of vaccination campaigns. It has been shown that rough lipopolysaccharide (RLPS) of *Brucella abortus* strain RB51 can be used to detect antibodies to rough *Brucella* spp. Nielsen *et al.* (2004) reported performance indices of developed indirect ELISAs using RB51 RLPS to detect antibody responses of cattle, sheep and dog to *B. abortus* strain RB51, *B. ovis* and *B. canis* which were 192.1, 196.3 and 195.8, respectively. In 2009, Robles *et al.* showed that homologous RLPS of RB51 was the best antigen in indirect ELISA for the detection of RB51 vaccine-induced serological responses in heifers over time. The RLPS of strain RB51 used in these studies was produced by phenol-chloroform-petroleum ether method, but no data about its characterisation were presented. It has also been suggested that due to batch-to-batch uniformity of RLPS from RB51 strain, it is the most useful antigen for serological diagnosis of *B. ovis* infection in sheep by ELISA (Nielsen *et al.*, 2007).

Hence, regarding the importance of RLPS from RB51 vaccine strain for diagnostic purposes, our study was aimed at characterisation of rough lipopolysaccharide from *Brucella abortus* strain RB51 extracted by phenol-chloroform-petroleum ether method which can further be used as the antigen to develop serological assays

for the identification of calves and cows vaccinated with RB51 vaccine in Iran.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Brucella abortus strain RB51 was obtained from CZ Veterinaria (Spain). Genus- and strain-specific PCR assays were performed on DNA of the bacterium extracted by boiling method to verify RB51 strain identity (Table 1). A suspension of the bacterial cells in Brucella broth (HiMedia, India) was cultured on potato dextrose agar (PDA) in Roux flasks which were then incubated for 72 hours at 37 °C. The cells were harvested into distilled water and checked for purity using blood agar (Merck, Germany) and tryptic soy agar (Merck, Germany) media. A sample of the harvested suspension was used to confirm the rough phenotype using standard methods including agglutination with 0.1% (w/v) acriflavine in distilled water and staining with crystal violet (Alton *et al.*, 1988). Harvested bacterial cells were washed for three times and then dried by acetone.

Brucella abortus strain 99 obtained originally from Animal and Plant Health

Agency (Weybridge, UK) was kept in our culture collection and used routinely to produce brucellosis diagnostic antigens at Razi Vaccine and Serum Research Institute. This strain was also cultured on PDA in Roux flasks and incubated for 72 hours at 37 °C. Bacterial cells were harvested in normal saline and checked for purity as mentioned before.

Preparation of rough lipopolysaccharide from strain RB51

RLPS of RB51 was extracted by phenol-chloroform-petroleum ether method (Galanos *et al.*, 1969). Eight grams of acetone-dried bacterial mass were mixed and homogenized in 250 mL of phenol-chloroform-petroleum ether mixture (2 volumes of 90% phenol + 5 volumes of chloroform + 8 volumes of petroleum ether) for 5 minutes at room temperature. The mixture was then centrifuged at 10,000 g at 4 °C for 30 minutes. The supernatant containing RLPS was separated. The cell sediment was extracted for two more times and the supernatants were pooled. Chloroform and petroleum ether were evaporated (Nielsen *et al.*, 2004). RLPS was precipitated in remaining phenol by adding 5 volumes of cold methanol containing 1 percent methanol saturated

Table 1. Primers and conditions of PCR assays used for identification of *Brucella abortus* strain RB51 in this study.

Primers	Target	PCR conditions	Amplification size
Genus-specific (Ouahrani-Bettache <i>et al.</i> , 1996): ISP1: 5'GGTTGTTAAAGGAGAACAGC3' ISP2: 5'GACGATAGCGTTTCAACTTG3'	IS711	Denaturation: 95 °C, 35 s Annealing: 56 °C, 45 s Extension: 72 °C, 45 s 30 cycles	~600 bp
Strain-specific (Vemulapalli <i>et al.</i> , 1999): F:5'GCCAACCAACCCAAATGCTCACAA 3' R:5'TTTAGTTTGCCGTAATATAGGTCTAGA ACCTGTC3'	<i>wboA</i>	Denaturation: 95 °C, 60 s Annealing: 59 °C, 60 s Extension: 72 °C, 90 s 40 cycles	~900 bp

with sodium acetate (Moreno *et al.*, 1979). The sediment was collected by centrifugation at 10000 g for 10 min and the supernatant was discarded. RLPS was then resuspended in distilled water and dialysed against distilled water for 3 days with frequent change of the water. It was finally lyophilised.

Preparation of hot-saline extract from S99

Hot-saline extract (HSE) of *Brucella abortus* strain 99 was used for SDS-PAGE along with RLPS from *Brucella abortus* strain RB51. Harvested suspension of bacterial cells in normal saline was autoclaved in 121 °C for 15 minutes. It was centrifuged at 10,000 g at 4 °C for 30 minutes (OIE, 2012b). The supernatant was separated and dialysed against distilled water. The extract was then lyophilised.

Limulus amebocyte lysate (LAL) assay

LAL assay was done using PyroMed Kit (SinaPharm, Austria) with single test tubes as manufacturer's instruction. A vial of lyophilised RLPS was reconstituted in 1 mL of water for injection (WFI). The reconstituted RLPS (200 µL) was added to a test tube. The same amount of the used WFI was added to another test tube as negative control. *Escherichia coli* LPS (supplied in the kit) was also used as positive control as instructed by the manufacturer. The test tubes were incubated at 37 °C for one hour. Positive result was determined visually as gelation and turbidity after incubation.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The method described by Apicella (2008) was applied with some modifications for SDS-PAGE of the reconstituted RLPS in distilled water diluted to contain 2 mg/mL

RLPS. Briefly, a 14% polyacrylamide gel in 1.88 M Tris-HCl buffer, pH 8.8, was used as resolving gel which did not contain SDS. The stacking gel was prepared by 5% acrylamide in 1.68 M Tris-HCl buffer (pH 6.8) having 0.1% SDS. The diluted RLPS was mixed with the same volume of Laemmli sample buffer consisting of 1 M Tris-HCl, pH 6.8 (1.25 mL), 10% SDS (4 mL), glycerol (2 mL), 0.5 M EDTA (0.5 mL), bromophenol blue (4 mg), 2-mercaptoethanol (0.2 mL) and distilled water (2.05 mL) (Laemmli, 1970). The mixture was then boiled for 5 minutes and loaded in the well. Simultaneously, HSE of *Brucella abortus* strain 99 was also loaded with the same concentration and using the same procedure. A constant current of 20 mA was applied for 2.5 hours. After termination of electrophoresis, the previously described LPS-specific staining method with silver nitrate was performed (Hitchcock & Brown, 1983; Apicella, 2008). The gel was fixed overnight in a solution containing 40% ethanol and 5% acetic acid in distilled water. One percent periodic acid was added to the solution 10 minutes prior to proceeding. The gel was then washed three times with distilled water for 10 minutes. The gel was placed in the freshly prepared staining reagent which was made by mixing 28 mL of sodium hydroxide, 3 mL of 25% ammonium hydroxide and 5 mL of 20% silver nitrate and adding 115 mL distilled water. After 10 minutes, it was washed with distilled water for 3 times. The gel was transferred into developer solution made of 10 mg of anhydrous citric acid, 0.1 mL of 37% formaldehyde and 200 mL distilled water, kept in the dark for 10 minutes. Finally, it was rinsed with water to stop reaction.

Agar gel immunodiffusion (AGID)

AGID was performed by 0.8% agarose gel in borate buffer (pH 8.3) containing 10% NaCl (OIE, 2012b). Lyophilised RLPS was reconstituted in phosphate buffered saline (PBS), pH 6.8, with 0.25% triethylamine. To produce positive serum against RLPS, 10⁹ RB51 bacteria were injected into a Dutch rabbit intravenously. Ten days later, the rabbit was euthanised and its blood was collected. Serum obtained from the same rabbit before injection of bacteria was used as the negative serum. The sera were kept at -70 °C until use. For the test, 50 µL of each of RLPS and sera were added to wells punched in the gel. The gel was incubated at room temperature in a humid chamber for 5 days. Appearance of precipitation line between RLPS and serum wells was considered as a positive reaction.

Evaluation of RLPS purity

To evaluate the purity of the RLPS obtained, DNA and protein contents of three different batches were determined before

lyophilisation by measuring UV absorbance using a NanoDrop® ND-1000 Spectrophotometer (USA) at 260 and 280 nm, respectively. As per manufacturer's instruction for protein quantification, an absorbance of 1 at 280 nm was considered equal to 1 mg protein per mL.

RESULTS

The DNA of *B. abortus* strain RB51 used in this study produced an expected band of about 600 bp in the *Brucella* genus-specific PCR targeting IS711 (Fig. 1A). The strain-specific PCR assay also amplified a 900-bp fragment as expected for RB51 strain (Fig. 1B). All harvested bacterial cells were in rough phase since 100% of the colonies were stained by crystal violet.

The extracted RLPS obtained by precipitation with cold methanol reagent in phenol followed by reconstitution and dialysis was insoluble in water. On average, 36.2 mg RLPS was produced from every gram of dried bacteria (a mean yield of 3.62 % of bacterial dry weight). As

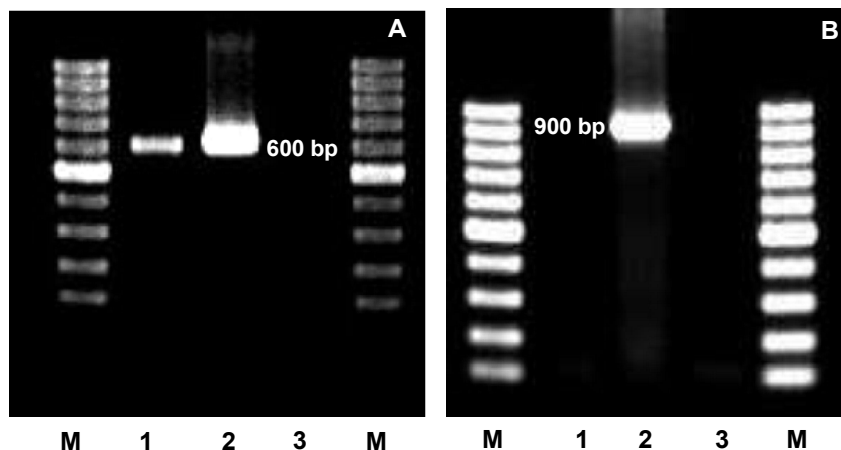


Fig. 1. Results of PCR assays proving the identity of *B. abortus* strain RB51. **A.** Genus-specific PCR targeting IS711; **B.** RB51 strain-specific PCR targeting *wboA*. M: Molecular weight marker (100 bp), lane 1: S99, lane 2: RB51, lane 3: negative control without template DNA.

shown on Fig. 2, prepared RLPS resulted in gelation and turbidity in the LAL assay performed using the commercial kit. This is noteworthy that a 1:1000 dilution of the prepared RLPS could also produce the same positive reaction (data not shown).

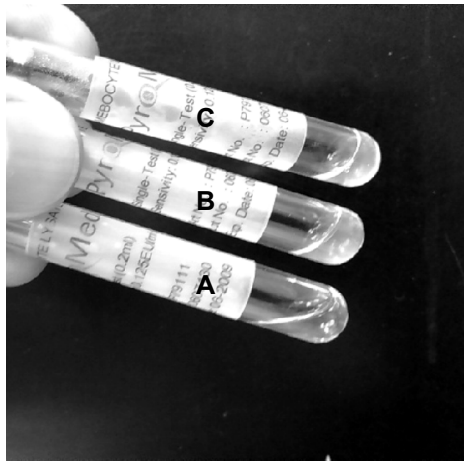


Fig. 2. Gelation in LAL assay as positive reaction by prepared RB51 RLPS. **A.** Negative control (WFI), **B.** Kit's positive control, **C.** Prepared RB51 RLPS.

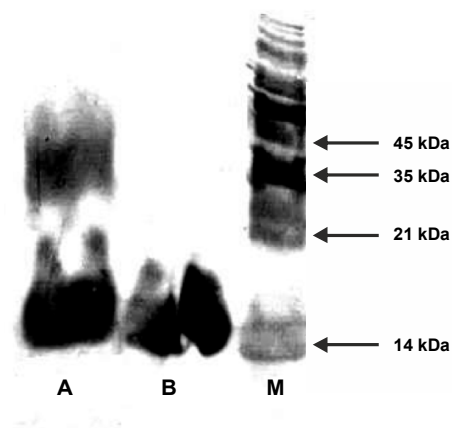


Fig. 3. SDS-PAGE profile of RLPS. **M:** Molecular weight marker, **A:** HSE from S99, **B:** RLPS from RB51 strain.

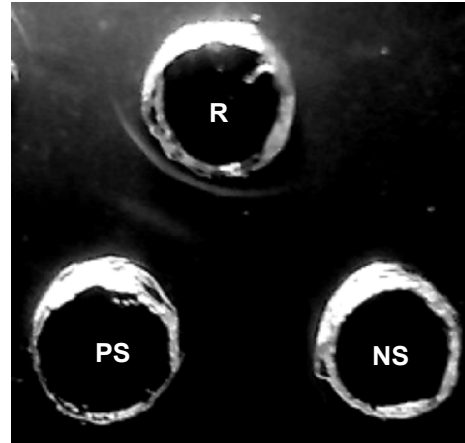


Fig. 4. Precipitation of RLPS from RB51 with specific serum in AGID. **PS:** rough-specific rabbit serum, **NS:** negative rabbit serum, **R:** RLPS from RB51 strain.

Analysis of the RLPS preparation by SDS-PAGE followed by silver-staining revealed presence of a single band of about 14 kDa. The HSE of *B. abortus* strain 99 showed another band of 35 to 45 kDa which can be attributed to the O-polysaccharide of its smooth LPS (Fig. 3).

Formation of a precipitation line was observed when using the RLPS against prepared RLPS-specific rabbit serum in agar gel double immunodiffusion (Fig. 4).

Protein and DNA concentrations (mean±SD) in RLPS preparation were 1.9±0.11 mg/mL and 145.6±5.6 ng/μL, respectively.

DISCUSSION

Bovine brucellosis remains an important enzootic disease in Iran which imposes economic losses on animal production industries and poses hazards to the public health. *B. abortus* biovar 3 is known as the most prevalent cause of bovine brucel-

losis in the country while other biovars and also *B. melitensis* have been isolated in different provinces (Zowghi *et al.*, 2008). Animal vaccination and test-and-slaughter have been the main strategies to combat brucellosis in cattle population of Iran according to National Brucellosis Control Program. Vaccination of calves and cows was done for decades using *B. abortus* S19 vaccine which, due to its smooth phenotype, interfered with serological diagnosis of infected animals and hence, placed limitations on simultaneous implementation of the test-and-slaughter campaign. Therefore, RB51 vaccine was introduced as a newly developed rough vaccine since 2004 to overcome this problem with S19 vaccine. Currently, calves are vaccinated with a standard dose of RB51 vaccine containing $10\text{--}34 \times 10^9$ bacteria at the age of 4–12 months, and reduced doses of the vaccine having $1\text{--}3 \times 10^9$ cells/dose are used for the immunisation of adult animals annually.

As other Gram-negative bacteria, *Brucella* lipopolysaccharide consists of lipid A, core oligosaccharide and O-polysaccharide (O-PS) (Moriyon *et al.*, 2004; Haag *et al.*, 2010). O-PS is a polymer of N-formyl-perosamine responsible for smooth phenotype in brucellae. Strain RB51 is a stable rough mutant lacking OPS which was approved for use in cattle in USA during the 1990s (Olsen & Stoffregen, 2005; Dorneles *et al.*, 2015). Strain RB51 vaccine has replaced S19 in many countries for active immunization of bovines against brucellosis (Avila-Calderon *et al.*, 2013). Successful use of RB51 vaccine as a complementary tool has been reported for the control of bovine brucellosis in different endemic countries (Martins *et al.*, 2009; Herrera-Lopez *et al.*, 2010; Sanz *et al.*, 2010; Saez *et al.*, 2014; Caetano *et al.*, 2016).

Since vaccination of calves and cows against brucellosis is done by the private sector in Iran, this is of paramount importance to have a practical test to assure accuracy of vaccination implementation by assessing humoral responses induced after vaccination. For this purpose, routine serological tests like Rose Bengal test are used in case of vaccination with smooth vaccine strains. However, due to the roughness of RB51 vaccine, these tests in which smooth antigens are used cannot be useful. Rough lipopolysaccharide of RB51 vaccine strain has been successfully utilised to develop serological test for detection of antibody responses to the vaccine strain (Nielsen *et al.*, 2004; Robles *et al.*, 2009). While hot phenol-water method has been used for the preparation of LPS from rough brucellae (Kreutzer *et al.*, 1979; Kreutzer & Robertson, 1979; Moreno *et al.*, 1979; 1984; Kianmehr *et al.*, 2015) including strain RB51 (Schurig *et al.*, 1991), phenol-chloroform-petroleum ether method was reported as a specific method for preparation of rough *Brucella* LPS (Jones *et al.*, 1973) and utilised by many other researchers for extraction and characterisation of LPS from rough *Brucella* mutants and strains (Freer *et al.*, 1995; González *et al.*, 2008; Fontana *et al.*, 2016). In addition, the RB51 RLPS used in development of ELISA assays for evaluation of post-vaccination serological responses to RB51 vaccine was prepared by the latter method (Nielsen *et al.*, 2004; Robles *et al.*, 2009). Therefore, we used phenol-chloroform-petroleum ether method for extraction of RLPS from *B. abortus* strain RB51 and since no information was available about characterisation of RB51 RLPS prepared by this method, immunochemical characterisation was done. Roughness and identity of the *B. abortus* strain RB51 used in

our study were verified by standard methods and PCR assays, respectively.

In our experiment, direct dialysis of phenol phase containing RLPS after evaporation of chloroform and petroleum ether as explained by Nielsen *et al.* (2004) and Robles *et al.* (2009) resulted in accumulation of RLPS in a small amount of phenol which remained inside the dialysis bag even after 5 days leading to poor yield in aqueous phase. Thus, we induced precipitation of RLPS in phenol phase by cold methanol reagent (Moreno *et al.*, 1979) which facilitated separation from phenol and increased our yield. Moreno *et al.* (1979) reported a yield of 0.008% of bacterial dry weight by a similar method for RLPS of *B. abortus* strain 45/20. In their study, one volume of water was added to the phenol making aqueous and phenol phases, but we directly precipitated RLPS in phenol without adding water. The higher yield in our study might be due to the bacterial strain or difference in precipitation method. The RB51 RLPS prepared after centrifugation, reconstitution and dialysis was insoluble in water making a fine cloudy mixture. Insolubility of RLPS from other rough brucellae was also reported previously (Jones *et al.*, 1973; Moreno *et al.*, 1984). Since limulus lysate gelation activity was reported before as an important marker for the presence of smooth and rough *Brucella* LPS (Moreno *et al.*, 1979), we used it to confirm RLPS extraction. As our result, the extracted RLPS could result in positive reaction in LAL assay which proves its presence. To the best of our knowledge, this is the first report which shows that RLPS from *B. abortus* strain RB51 had limulus amebocyte lysate gelation activity.

RLPS of RB51 strain was visualised as a band of about 14 kDa after SDS-PAGE and LPS-specific silver staining. This re-

sult is similar with the previous study by Schurig *et al.* (1991) in which RB51 RLPS was extracted by hot phenol-water method. For HSE from S99, while the same size band was present, another band was also observed with molecular size more than 35 kDa which can be due to the OPS of this smooth strain (Moreno *et al.*, 1979; Schurig *et al.*, 1991). As shown by the formation of a precipitin line, our extracted RLPS also reacted with the specific rabbit serum in AGID after solubilisation with triethylamine while was not precipitated by negative serum suggesting its specific reactivity. This finding reveals immunological reactivity of the prepared RB51 RLPS with specific antibodies making it suitable for use in diagnostic assays. Determination of protein and DNA concentrations in our RLPS preparation showed that while the DNA content was negligible, there was a protein impurity of more than 30%. This can make it required to add a step for purification of RLPS from proteins. However, further investigations are necessary to evaluate the effects of protein impurities in diagnostic assays developed by this RLPS preparation.

In conclusion, our experiment showed successful extraction and preparation of rough LPS from *B. abortus* strain RB51 by phenol-chloroform-petroleum ether method which was characterised immunochemically by LAL assay, SDS-PAGE and AGID.

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