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A Simple Method for Extraction of Lipopolysaccharides from *Brucella Melitensis*

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Abstract

Background: Brucellosis is the most common zoonotic bacterial disease around the world, and its causative agent is *Brucella,* a gram-negative bacterium. Lipopolysaccharide (LPS) molecules are the most significant surface antigen of *Brucella* bacteria. This antigen is used in a high number of research and diagnostic fields. The present study was designed in order to introduce a simple method to extract LPS from *Brucella melitensis*. **Materials and Methods: S**mooth strains of *B. melitensis* were cultured in large quantity, and LPS was extracted through changes in the hot-phenol method. The LPS concentration was measured by using the dimethyl-methylene blue method. The contamination rates of proteins and nucleic acids were determined using the bicinchoninic acid (BCA) method and absorbance measurement at 260 nm, respectively. **Results:** The amount of the extracted LPS was 1.1% of the wet weight of the bacteria. The rate of contamination with nucleic acid was measured to be 0.2% of the LPS. The protein contamination was not detectable through the BCA method. **Conclusion:** The advantages of our newly-introduced method are considerable decrease in the time required to extract LPS; independence from nucleic acid and protein digestive enzymes; an extraction rate of LPS equivalent to other applied methods; nucleic acid contamination equal or lower than other methods, and no protein contamination; and independence from equipment such as ultracentrifuge and chromatography. **[GMJ.2021;10:e1944]** **DOI:10.31661/gmj.v10i0.1944**

Keywords: *Brucella melitensis*; LPS Extraction; Hot-phenol

Introduction

Brucellosis is the most common zoonotic bacterial disease in the world, which infects over 500 thousand individuals annually, causing considerable economic and health issues [1]. *Brucella*, a gram-negative bacteria, is the cause of this disease, and the major pathogens in humans are *Brucella abortus* and *B. melitensis,* which can cause acute and chronic disease[2]. As the non-specific clinical symptoms of this disease have a high overlap with other infectious and non-infectious diseases, this disease's diagnosis is difficult, and it is

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labeled as the disease of mistakes[1]. Lipopolysaccharide (LPS) molecules are the main and most significant surface antigen of *Brucella* bacteria [2] and are essential for physical integrity and the performance of the outer membrane. LPS commonly covers the outer membrane in all gram-negative bacteria, which acts as one of the targets of the mammalians' immune system. LPS can attach to its antibodies and activate lymphocytes, macrophages, and granulocytes [3, 4]. Since LPS is the most significant antigen of *Brucella's* outer membrane and elicits a dominant immune response, it is assumed that LPS is one of the most remarkable factors contributing to brucellosis immunogenicity [5]. Structurally, LPS or endotoxins are phosphorylated glycolipids composed of three parts that are the outer polysaccharides (o-chain) being the outermost part and composed of repetitive heptode polymers interfering in the bacterial antigenicity, the core that is significant in membrane permeability, and the lipid A being the innermost part anchoring the LPS into the membrane [6, 7]. Given the importance of LPS in these bacteria, LPS is applied in various clinical and experimental studies. One of the most important applications of LPS is in clinical laboratories where diagnosing infection caused by *Brucella*. The enzyme-linked immunosorbent assay (ELISA) kits have been designed as a high-sensitivity serologic test using this antigen [8]. Since the O-chain part in LPS is a part inducing an immune response, it is used to induce antibody production in laboratory animals as well as to the design of serologic tests based on the diagnosis of produced antibodies against this part of LPS. As LPS is a potent stimulant of the immune response, it has been used in vaccine production to prevent the incidence of brucellosis [9]. Considering that LPS has extended applications in various research fields, it is necessary that an appropriate and simple method is proposed to extract LPS from *Brucella*. Various methods have described extracting this antigen from *Brucella* variants, which have mainly been based on the hot-phenol method, while it has advantages and/or disadvantages. Some of the disadvantages of the existing methods are being time-consuming, requiring multiple stages and advanced equipment, and applying multiple enzyme steps [10, 11]. Regarding the fact that the majority of the conducted studies have been on *B. abortus* and the necessity for a simpler method to extract LPS from *Brucella* bacteria, the present study was designed based on a combination of the existing methods of LPS extraction from *Brucella* bacteria to simplify the process of LPS extraction from *B. melitensis*.

Materials and Methods

Bacterial Strain and Growth Conditions

Acute *B. melitensis* bacteria biotype one strain was supplied from the archive of the Brucellosis Section at Razi Vaccine and Serum Research Institute (Karaj, Iran) and following the re-identification by using the phage typing method, they were cultured on Brucella agar culture medium in 30 plates and incubated at 37°C for 48 hours. Then, the bacteria on the plates' surface were washed by employing an inoculation needle with 3-5 ml of Brucella broth added by using sterile pipettes into the dishes within a class 3 biosafety cabinet. Subsequently, the Brucella broth liquid containing the washed bacteria was obtained in a sterile glass container. According to the Alton method, the bottle containing the bacterial suspension was placed at 80°C water bath for 30 minutes to kill the bacteria after checking its purity and homogeneity. Afterward, in order to be certain that the bacteria are killed, the liquid containing the bacteria was cultured on Brucella agar culture medium. When the bacteria were observed to be non-growing, the bacterial suspension was transferred into 250 ml buckets with twistable caps under sterile conditions and centrifuged at 4 degrees for 30 minutes at 12000g. The obtained sediments of the bacterial strains were transferred to sterile tubes under sterile conditions and transported to Kerman University of Medical Sciences.

Extraction of LPS from B. melitensis through Optimized Hot-Phenol Method

The extraction of LPS from the bacteria was performed based on the hot phenol method [10] with some modifications. Briefly, the wet bacteria weighing 30, 50, 75, and 100 mg were fully dissolved in 510 µl of distilled

water, and then this cellular suspension was heated to reach 68°C. 570 µl of 90% phenol preheated to reach 68°C was added to the suspension and stirred for 30 minutes at the mentioned temperature. Subsequently, the sample's temperature was quickly dropped to 5°C on an ice bath and then centrifuged at 4°C for 15 minutes at 8000 g. Consequently, four phases were formed as follows: 1) the top phase: phenol-saturated aqueous layer; 2) between aqua and phenol in the form of white sediment; 3) the third phase under the white sediment: aqua-saturated phenol; and 4) the sediment formed on the bottom of the tube.
The top aqueous phase and the underneath sediment were carefully separated and discarded. The phenol phase was carefully separated, and in order to deposit the nucleic acids and proteins, a half volume of cold methanol was added to it (unlike most bacteria, LPS enters the phenol phase in *Brucella*) [12]. After adding the cold methanol, the samples were placed at 4°C for 30 minutes and then centrifuged at 1500g for 10 minutes at 4°C. The top phenol phase was separated, and the protein and nucleic acid sediments were discarded. During the next stage, and to deposit the remaining protein contamination, 50 mg of trichloroacetic acid per mL of the solution was added and stirred for 15 minutes at 56°C. It was subsequently centrifuged at 1500g and a temperature of 4°C for 10 minutes and the supernatant was separated, and the protein sediment was discarded. In order to deposit the LPS, three volumes of methanol reagent (99 volumes methanol+1 volume sodium acetate-saturated methanol) were added and stirred on ice for one hour. The LPS was deposited through centrifuging at 8000g for 20 minutes at 4°C degrees, and the supernatant was discarded. To eliminate the remaining phenol, the sediment was dissolved in 25 µl of distilled water and then 75 µl of cold methanol reagent was added, shaken for one hour at room temperature, centrifuged at 8000g for 20 minutes at 4°C. The obtained sediment was dissolved in 500 µl of distilled water, and its concentration was measured by the dimethyl-methylene blue (DMB) method [13]. Ultimately, to conduct the final confirmation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 14%) was conducted, and the LPS product was evaluated by silver nitrate staining. The designed LPS extraction method is shown in Figure-1 schematically.

Determining the Concentration of the Extracted LPS
In order to determine the concentration of the extracted LPS, standard LPS of *Salmonella typhimurium* (L6511) and dimethyl methylene blue-based reagent were employed. To achieve a final volume of 100 mL, the reagent was prepared with 1.6 mg dimethyl methylene blue stain, 428 mg glycine, 333 mg NaCl, 4.7 mL 1-N NaOH, and 0.5 mL ethanol 80%. The described compounds were reached the volume of 100 mL and stirred for 5 hours to reach homogeneity. The pH of the obtained solution was 10.1.

Measuring the Nucleic Acid Contamination
In order to measure the nucleic acid contamination, the absorbance of the extracted sample was read in 260 nm against distilled water as the blank. The contamination rate was described as a percentage of the extracted LPS.

Measuring the Proteins Contamination
The bicinchoninic acid (BCA) method was used to determine the protein amount in the extracted LPS. The standard curve was drawn by using albumin as standard.

Optimizing the Ratio of the Wet Bacteria Weight to the Utilized Solutions
To optimize the bacterial weight ratio to the utilized solutions to obtain the maximum amount of LPS, with the fixed amounts of extraction solutions, varying amounts of the bacteria were used to extract LPS. Subsequently, the LPS concentration and the contamination with nucleic acids and protein were measured in the case of each weight.

Results

Determining the Concentration of the Extracted LPS
The standard curve was drawn through standard LPS of *S. typhimurium,* and the obtained concentration after LPS extraction is depicted as follows (Figure-2 and Table-1). The

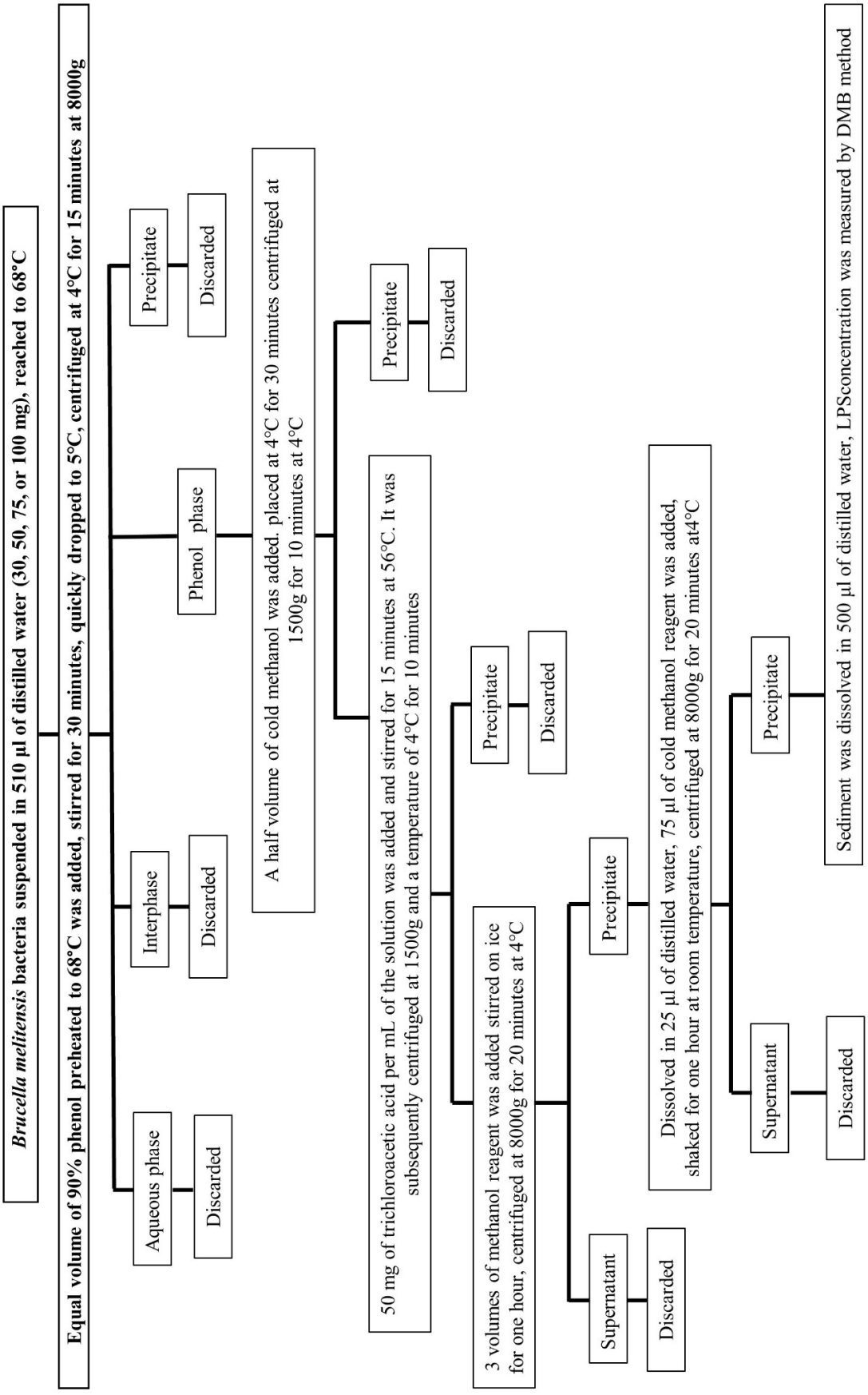

Figure 1. The designed LPS extraction method is shown schematically.

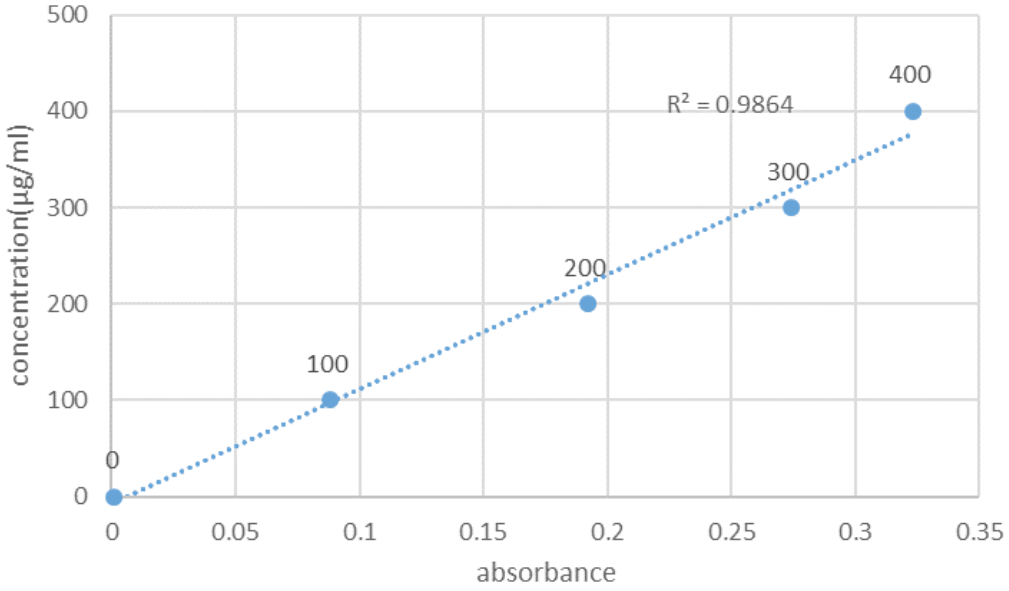

Figure 2. The standard curve is drawn trough standard LPS of *S. typhimurium* and by using the DMB reagent. The standard curve was repeated three times. The linear range is up to 400 µg/mL in measuring the LPS concentration by using the DMB reagent [13]. The high-concentration samples were diluted, and their concentration was re-measured.

Table 1. Optimizing the Desirable Bacterial Weight to a Fixed Volume of the Utilized Solutions to Obtain the Maximum Amount of LPS.

Sample	wet bacterial weight(mg)	Extracted LPS amount(µg)	Percentage of the LPS extracted from wet bacterial weight	Rate of contamination with nucleic acids (percentage from LPS concentration)
1	100	335	0.33%	2.6%
2	75	610	0.81%	1.5%
3	50	550	1.1%	0.2%
4	30	265	0.88%	2.9%

amount of the extracted LPS was 0.3 to 1.1% of the wet bacterial weight.

Measuring the Contamination with Nucleic Acids

The nucleic acid contamination was measured at 260 nm. The concentration of the nucleic acids was estimated to be 0.2 to 2.9% of the concentration of the extracted LPS. Table-1 demonstrates the nucleic acid contamination rate as compared to the extracted bacterial weight. The absorbance range of the extracted LPS samples is illustrated in Figure-3. The absorbance range of the LPS was at wavelengths lower than 230 nm. As depicted in Figure-3, the maximum absorbance was at ranges lower than 230 nm, which pertains to LPS, and no significant absorbance can be observed in 260 and 280 wavelengths confirming the absence of contamination with nucleic acids and proteins.

Optimizing the Ratio of Wet Bacterial Weight to the Utilized Solutions

The desirable obtained weight to reach the maximum amount of LPS was equal to 50 mg of the wet bacteria. As demonstrated in Table-1, in weights lower or higher than 50 mg, the amount of the extracted LPS was lower, and the contamination with nucleic acids was higher.

Measuring the Contamination with Proteins in the Extracted Samples

The amount of protein contamination was measured with the BCA method. The amount

of proteins was no significant and indeterminable with the BCA method.

Electrophoresis of the LPS on the SDS-PAGE
The electrophoresis of the extracted LPS with the standard LPS of *S. typhimurium* was conducted as shown in Figure-4.

Discussion

LPS molecules are considered as the most significant surface antigen of gram-negative bacteria, including *Brucella,* and it can be applied in different fields due to specific antigenic characteristics. A growing number of

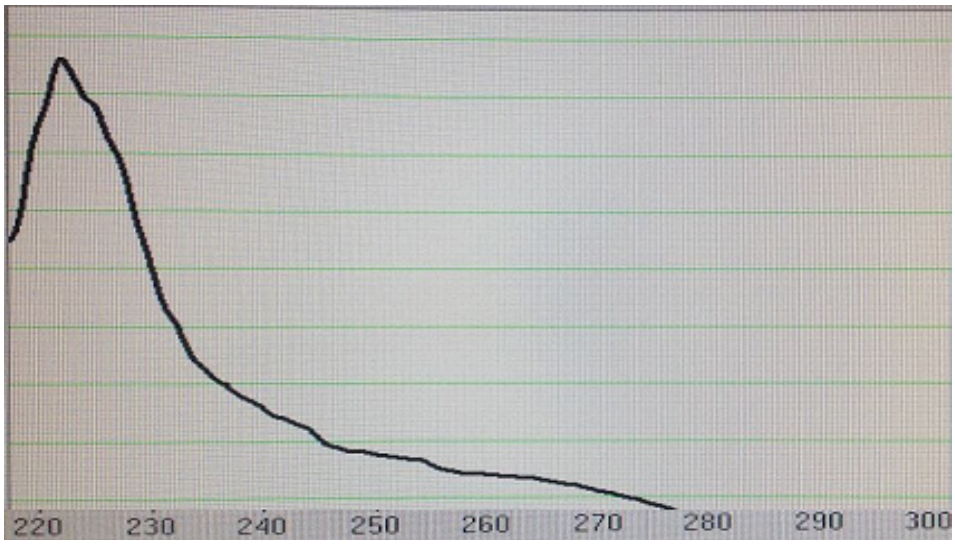

Figure 3. The absorbance spectrum of the extracted LPS from *B. melitensis* bacteria. The depicted peak at wavelengths between 220 and 230 pertains to LPS molecules. The wavelength pertaining to nucleic acids is 260 nm and 280 nm in the case of proteins.

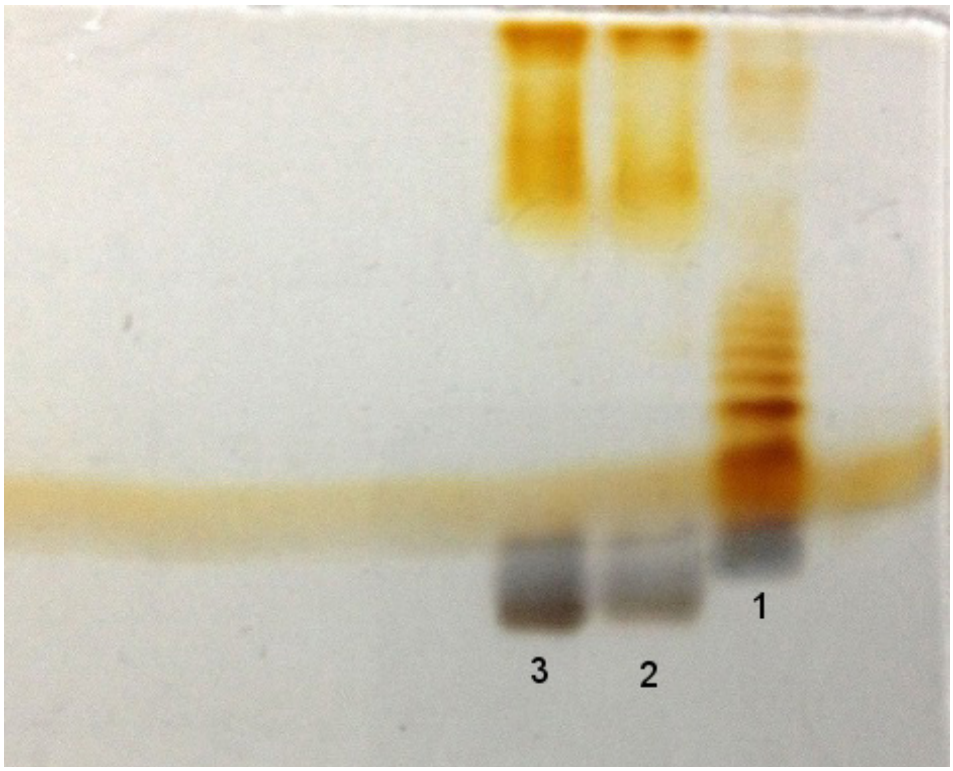

Figure 4. Electrophoresis of the LPS with silver nitrate staining. The standard LPS of *S. typhimurium* with a concentration of 0.5 mg/mL (1) and the extracted LPS from *B. melitensis* bacteria with a concentration of 0.5 (2) and 1 mg/mL (3). As depicted, following the silver nitrate staining, the band pertaining to the LPS from *B. melitensis* with varied densities appeared similar to previous studies [20].

established evidence has been conducted on the LPS extraction from gram-negative bacteria, such that the majority of them utilize an optimized hot-phenol method to extract LPS [10-12, 14]. The hot-phenol method was firstly introduced by Westphal and Jann (1965), and it is currently referred to as the Westphal method [15]. Unfortunately, LPS of *B. melitensis* bacteria have not yet been commercially available, and those researchers who tend to conduct studies on the case of LPS molecule have to extract it using a proper method. Selecting a proper LPS extraction method depends on the type of bacteria and the available equipment for extraction. Concerning the hot-phenol method, for instance, unlike other bacteria, the LPS of *Brucella* bacteria enters the phenol phase instead of the aqueous phase though many of the existing protocols on LPS extraction have not been mentioned this point [12, 14]. The studies on LPS extraction from *Brucella* are mainly conducted on *B. abortus*. In a method by Baker and Wilson [16, 17], a crude extract of LPS was ultimately obtained from *B. abortus* through the hot-phenol method, which required further purification of LPS from this crude extract. Similarly, in another method on *B. abortus*, the obtained unrefined extract through Baker and Wilson method was used for LPS purification. Despite being successful, it was highly time-consuming and required repeated centrifuges, dialyzes, and chromatography procedures [10, 16, 17]. Fewer studies have been conducted on extracting LPS from *B. melitensis*. In this regard, one instance is Van De Verg *et al*. (1996) method. In this method and to extract the LPS, two stages of centrifuge at 105000g and each time for 5 hours were conducted, which was time-consuming and required equipment such as ultra-centrifuge [11]. The other methods required several protease enzymes, DNase, RNase, and long incubations and overnights duration [14]. As mentioned, considering the drawbacks of the applied methods for LPS extraction from *Brucella,* including being time-consuming, requiring equipment such as ultra-centrifuge, chromatography, and various digestive enzymes in order to eliminate contaminations [10, 11, 14], the present study was designed to facilitate the extraction of this antigen. The basis of LPS extraction in this study was an optimized hot-phenol method proposed by Moreno *et al*. for LPS extraction from *B. abortus* bacteria [10]. By combining other applied methods to extract LPS from *Brucella* and applying alterations to those methods, we introduced a more straightforward method to extract LPS from *B. melitensis* [18]. In the present study, the amount of the obtained LPS relative to the bacterial weight has been similar to the previously studied to some extent. For instance, the extraction rate of *B. abortus* in the studies using different methods ranged from 0.2 to 1.3% of the wet bacterial weight estimated to be approximately 1% in the present study. The LPS concentration measured using the DMB reagent indicates a linear range of up to 400 µg/mL. Hence, it is necessary that diluting be conducted in case of higher densities of LPS [13]. To investigate the purity of the extracted LPS and comparing it to similar extraction methods, the amount of protein and nucleic acid was measured in the LPS samples. The contamination rate of proteins was indeterminable with the BCA method, which indicates our method's priority in eliminating protein contamination without utilizing protease enzymes. The contamination rate of nucleic acids showed a range between 0.2 and 2.9% of the obtained LPS. The absorbance spectrum drawn using nanodrop confirmed the no significant contamination with proteins and nucleic acids. Findings obtained from previous studies indicated that the contamination rates of proteins and nucleic acids among similar methods have been lower than 2.9 and 1.4%, respectively. The nucleic acid and protein contamination in our study were lower than this amount (0.2% of LPS for nucleic acid contamination and not detectable for proteins) which indicate the more success of our method to eliminate these contaminations compared with other reports [18, 19]. To optimize the ratio of the obtained bacterial weight to the utilized solutions to extract the maximum amount of LPS, by maintaining the number of reagents and solutions, varying amounts of the bacteria were obtained, and the extraction stages were conducted on them. The desirable obtained weight to extract the maximum amount of LPS was equal to 50 mg of wet bacteria. In higher and

lower amounts, lower amounts of LPS than the bacterial weight are obtained, leading to bacteria wastage and utilized materials. Considering the high value of the bacteria due to the risks during the culturing process and a requirement for special culturing equipment and trained staff, this optimization is of great significance. Moreover, the disproportion between the number of bacteria and the volume of solutions can increase nucleic contamination acids for undetermined reasons. Considering the numerous similarities between different types of *Brucella* bacteria, theoretically, this method can be possibly applied to extract LPS from other strains of *Brucella* with slight alterations, requiring further studies to confirm its success in the case of the other strains.

Conclusion

The modifications of LPS extraction from *B. melitensis* bacteria have not been yet investigated as of present. Based on the best of the authors' knowledge, the present method has been proposed for the first time. A considerable reduction in the time of LPS extraction, independence from nucleic acids and proteins digestive enzymes, extracted amounts of LPS equal to other methods, nucleic acid contamination equal or lower than other methods, lack of protein contamination, independence from the equipment such as ultra-centrifuge and chromatography, and a need for a simple centrifuge and a simple spectrophotometer instrument are among the most significant advantages of the present method. Moreover, considering the similarities between different strains of *Brucella*, this method might be applicable in other strains with slight alterations.

Acknowledgment

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Conflict of Interest

The authors declare that they have no conflict of interest.

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