Research Journal of Biological Sciences 3 (6): 576-580, 2008
ISSN: 1815-8846

Optimization of *Brucella abortus* S99 Lipopolysaccharide Extraction by Phenol and Butanol Methods

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Abstract: *Brucella abortus* S99 Lipopolysaccharide (LPS) extracted and purified by phenol and butanol methods after submerged culture in the fermentor and then the optimization of these two methods carried-out by some modifications to increase the yield of extraction and LPS content and also to decrease the protein and nucleic acid content of extracted samples. Extracted samples chemically characterized by LPS and Ketodeoxyoctanate (KDO) content measurement and the evaluation of protein and nucleic acid contamination. LPS and KDO content of optimized phenol extracted sample have been more than other extracted samples (146 EU mL^{-1} and 1.8%, respectively) and protein contamination of this sample has been less than the sample extracted by phenol (Westphal and Jann) method (2 versus 2.7%). Optimization of butanol method led to higher content of LPS and KDO in comparison with butanol (Morrison and Leive) method. Finally among four evaluated extraction methods (butanol, phenol, optimized butanol and optimized phenol), the optimized phenol extraction of *Brucella abortus* S99 lipopolysaccharide reported as the best method for industrial purposes (e.g. antigen production for diagnosis of brucellosis) based on chemical characterization of extracted samples.

Key words: *Brucella abortus*, lipopolysaccharide, optimization, extraction, phenol and butanol methods

INTRODUCTION

Brucellae are gram-negative, facultative intracellular bacteria that can infect many species of mammals and also humans. At least six species are recognized within the genus *Brucella*: *B.abortus, B.melitensis, B.suis, B.ovis, B.canis and B.neotomae*. This classification is mainly based on pathogenicity and hosts of these genuses (Corbel, 1997*)*. *Brucellae* cause Malta fever or brucellosis which is a zoonosis. The disease is found globally, but is highly reported from the Middle East including Persian golf zone, Mediterranean countries, India, North Africa and in parts of Mexico, Central and South America. This infectious disease represents a serious health problem and high amounts of industrial and economic disadvantages in the endemic areas (Corbel, 1997). 500000 new cases of infection are reported from all over the world and based on World Health Organization this statistic report is underestimating the real figure of newly established infections and is not a sharp data.

During the process of *Brucellae* pathogenesis, the organism may pass natural immunity barriers and enter the cytoplasmic space of phagocytic cells specially macrophages and then proliferate within host immune cells. *Brucellae* benefit from different potent mechanisms to achieve intra-cellular survival. In brief, these mechanisms are known as: inhibition of phagosome-lysosome fusion (inhibition of phagolysosome formation), inhibition of pH reduction within phagolysosomes, inactivation and clearance of reactive oxygen and nitrogen intermediates and substances produced by phagocytic cells, destruction of phagosome membrane and escape to the cytoplasmic space (Lapaque *et al*., 2005). Following *Brucella*-host interaction and immune responses to *Brucella* immunogenic components (e.g. LPS) brucellosis may be established as a chronic or acute infection.LPS is known as the main virulence factor of *Brucella* and LPS deficient strains have less virulence and intra-cellular survival potency. LPS is the main antigenic and immunogenic structure on the surface of

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Brucella (De Bagues *et al*., 2005; Lapaque *et al*., 2005). Among *Brucella* LPSs, the LPS of *B.abortus* has been extensively evaluated and studied. Different strains of *B.abortus* are classified to rough and smooth based on the LPS they express (rough LPS and smooth LPS, respectively). Wild type *Brucellae* mainly express smooth LPS (S-LPS). *B.abortus* S-LPS (as same as the most gram negative bacteria) consists of three domains: Lipid A, Core oligosaccharide and O specific polysaccharide (O-antigen), but possesses a peculiar non-classical LPS as compared with LPS of Enterobacteria such as *E.coli*. The lipid A of *B.abortus* LPS consists of special fatty acids (different from the other gram negative bacteria) and a unique structure. This moiety of *B.abortus* LPS possesses a diaminoglucose backbone and the acyl groups are longer (C18-19, C28) and are only linked to the core by amide bounds. Further the O-antigen of *Brucella* LPS is a homopolymer of perosamine (4, 6-dideoxy-4-formamido-d-mannopyranosyl) (Carmichael, 1990). Since, LPS is the main expressed antigen on the surface of *Brucella* the serologic responses following *Brucella* infection are directed against LPS. Thus in human and animal, the serologic diagnosis of brucellosis is usually based on the detection of specific anti-LPS antibodies (Wright and Neilson, 1990). The most current diagnostic assays for the detection of brucellosis, identification of host immune response conditions and differentiation chronic from acute infections are: tube seroagglutination or Wright assay, Slide seroagglutination or rapid Wright test, rose Bengal test, complement fixation assay, anti-globulin or Coombs-Wright test (Alton *et al*., 1988; Wright and Neilson, 1990). The applied antigen in above-named assays is almost LPS of *Brucella*.

Nowadays the application of *Brucella* LPS is a hot topic and extensively studied subject to design new diagnostic methods (such as candidate antigen to design new ELISA kits). LPS is also one of the major vaccine candidates for human brucellosis and also may be applied as an adjuvant to boost immune responses against other components. According to this large scale and industrial need to LPS of *Brucella*, it seems to be necessary to define an optimized method for the extraction and purification of *Brucella* LPS.

In the present study, we have initially extracted the LPS of *B.abortus* S99 (S-LPS) through two classic and well-known methods (Westphal and Jann, 1965; Morrison and Leive, 1978) and then through our optimized methods based on these classic methods. Finally, we comparatively evaluated chemical characterizations of our samples to define the best method to extract LPS of *B.abortus* S99 in the industrial scales.

MATERIALS AND METHODS

Bacterial strain, culture and fermentation conditions: *B.abortus* S99 obtained from the Collection of Standard Bacteria, Pasteur institute of Iran. This strain of *B.abortus* (biovar1) is smooth and CO2-independent. It was cultured in slant *Brucella* agar medium (Merck) at 37±1°C for 72 h and then cultured in a 5 L flask containing 2 L *Brucella* broth (Merck) under aeration by a sparger at 37±1°C for 72 h to achieve the seed culture. Seed culture inoculated to the 60 L industrial fermentor (Novapaljas, contact-flow B.V, Netherlands) with 40 liters working volume (the ingredients of fermentation medium are shown in the Table 1). The temperature and pH controlled at 37±1°C and 6.4, respectively. Finally after 60 h, fermentation process stopped by adding 10% phenol to the fermentation culture and biomass of *B.abortus* S99 harvested by centrifugation. During the fermentation process, cells were checked for purity and smoothness in 2 h periods by microscopic methods. The optical density of fermentor culture measured in 3 h periods after the initiation of fermentation process, in comparison to blank culture (measured before the inoculation of seed culture) to evaluate the growth of organisms according to standard curve of organisms growth (Fig. 1).

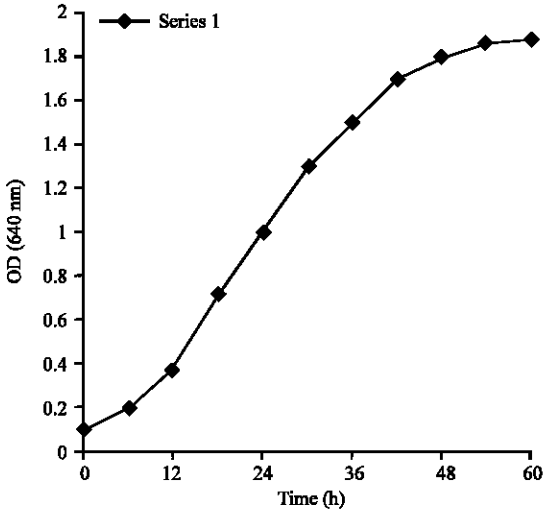

Fig. 1: Variation of biomass concentration during fermentation process

Table 1: The ingredients of fermentation medium

Ingredients	Concentration (g L^{-1})
PDAT	15
PDC	15
Sodium bisulphate	0.10
Yeast extract	10
Glucose	1-30
Peptone	30

LPS extraction procedure: *B.abortus* S99 S-LPS extracted by following four different methods.

Butanol extraction (Morrison and Leive, 1978) : Four hundred milliliter water-saturated n-butanol (99%) added to 50 g wet weight of *B.abortus* S99 at 4°C and then the aqueous phase treated as will be described for enzymatic digestion of proteins and nucleic acids:

Initially proteinase K (50 μg per 10 mg protein) and then both DNase and RNase (50 μg per 1mg nucleic acid) added to extracted samples to reduce protein and nucleic acid contaminations.

The amounts of used enzymes determined after the measurement of contaminations. The measuring methods will be described in chemical analysis part of this study.

Phenol extraction (Westphal and Jann, 1965): A total of 170 mL distilled water and then 190 mL of 90% (v/v) hot phenol (66°C) added to 50 g wet weight of *B.abortus* S99. LPS in the resultant mixture precipitated by cold methanol (4°C) and dissolved in 0.1 M Tris buffer. In order to reduce protein and nucleic acid contaminations, sample treated for enzymatic digestion as described in the previous method.

Optimized butanol extraction: This method is based on the first described extraction method and is an optimized method. The aim of this modification and optimization is the reduction of protein and nucleic acid contaminations as well as obtaining more LPS and KDO content in the extracted sample.

Total 400 mL of PBS buffer and then 400 mL of water-saturated n-butanol (99%) added to 50 g wet weight of *B.abortus* S99. The mixture shacked for 15 min at room temperature, then centrifuged at 4°C and the aqueous phase separated. The pellet dissolved in 400 mL of PBS buffer and centrifuged. The resultant aqueous phase added to the previously obtained one. LPS in the aqueous phase precipitated by cold (-20°C) pure (100%) ethanol (Merck) and sample kept at 4°C for 48 h to increase the precipitation of LPS. In the next step, sample centrifuged again at 4°C and the LPS-containing pellet dissolved in 100 mL distilled water. Finally 4 g of Trichloroacetic Acid (TCA) added and samples centrifuged to facilitate protein precipitation. The last step was the dialysis of extracted samples against deionized water for 72 h.

Optimized phenol extraction: A total of 170 mL distilled water and then 190 mL of 90% (v/v) hot phenol (66°C) added to 50 g wet weight of *B.abortus* S99 and mixture shacked for 15 min at 66°C. The temperature of sample suddenly decreased to 0°C and then centrifuged at 4°C. After centrifugation there were two LPS-containing phases: aqueous phase and phenol phase. LPS extracted of both phases by the addition of 25% and 100% (v/v) cold ethanol (4°C) to aqueous and phenol phases, respectively and then samples centrifuged. After this centrifugation step, supernatant and pellet separated for further purification process from aqueous and phenol phases, respectively. One hundred milliliters of 75% (v/v) cold (4°C) ethanol added to the supernatant of the aqueous phase and kept at 4°C for 6 h, then centrifuged and LPS-containing pellet dissolved in distilled water. Resultant pellets of phenol phase dissolved in distilled water and after 6 h centrifuged and LPS-containing supernatant kept for the final purification. Then both DNase and RNase added to extracted sample to reduce nucleic acid contamination.

Finally, 1 g TCA per 20 mL of aqueous and phenol samples added and kept at 4°C for 3 h. Finally, TCA treated samples centrifuged and dialyzed against deionized water for 72 h.

Chemical characterization of extracted samples

Protein content of extracted samples measurement: Protein contamination of extracted samples measured by Lowry method as described previously (Lowry and Rosebrough, 1951).

Nucleic acid content of extracted samples measurement: Nucleic acid contamination of extracted samples determined by measuring A-260.

Ketodeoxyoctanate of extracted samples measurement: KDO content of extracted samples (KOD is a unique component of LPS) measured by Weisbach method as described previously (Weissbach, 1959).

LPS concentration of extracted samples measurement: LPS concentration assayed by chromogenic Limulus Amebocyte lysate method (Texas A and M University Press, College Station. Bacterial Endotoxins Test, USP 26 NF 21. 2003).

SDS-Polyachrile amide gel electrophoresis of sample extracted by optimized phenol method: Electrophoretic pattern of the sample extracted by optimized phenol method determined by SDS-PAGE (14% and containing urea 4M) and bounds of LPS stained by AgNO3.

RESULTS

Fermentation process: The yield of fermentation process due to obtaining the biomass of *B.abortus* S99 has been satisfactory as the cell density and viable count of cells

Table 2: Chemical characteristics of extracted and purified LPS samples LPS

LPS	LPS concentration (Eu mL^{-1})	KDO content (%)	Protein content (%)	Nucleic acid content (%)
Extracted by butanol method	71	%1	<2	<1
Extracted by phenol method	86	13	2.7	<1
Extracted by optimized butanol method	83	13	<2	1.3
Extracted by optimized phenol method	146	18	<2	<1

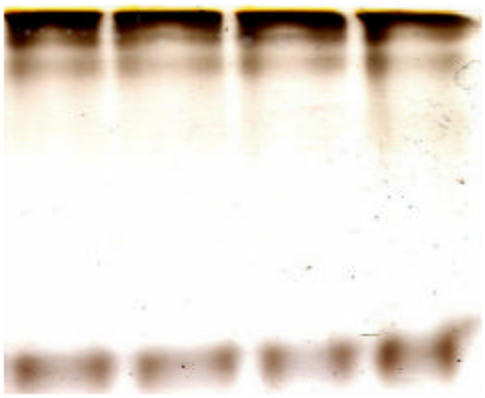

Fig. 2: Electrophoretic pattern of *B.abortus* S99 LPS sample, extracted by optimized phenol method

has been 8.9% and 3.1×10^{11}, respectively. There were no contaminations with other microorganisms in the harvested *B.abortus* S99 cells and microscopic evaluation of microbial content of fermentor, which has been performed at the end of fermentation process verified the purity of resultant biomass obtained by fermentation.

Chemical characterization: Protein contamination of 4 extracted samples (extracted by butanol extraction, phenol extraction, optimized butanol extraction and optimized phenol extraction) has been <2, 2.7, < 2 and <2%, nucleic acid contamination has been <1, <1, 1.3 and 1.4%, KDO content has been 1, 1.3, 1.3 and 1.7% and LPS concentration has been 71, 86,83 and 146 EU mL^{-1}, respectively (Table 2).

Electrophoretic pattern of *B.abortus* S99 LPS sample extracted by optimized phenol method, has been totally different from the pattern of classic LPSs such as enterobacterial LPS (e.g.*E.coli* or *Salmonella typhimurium*) and migrated as a smear (Fig. 2).

DISCUSSION

The variation of biomass concentration during fermentation process, evaluated by measuring the optical density of fermentor content, shows a logarithmic curve and would be compared to the standard curve of bacterial growth (Fig. 1). When we stopped the fermentation process (after 60 h), organisms were at the end of logarithmic phase of growth or at the primary stationary phase. If the process continues more than 60 h, the count of organisms will be decreased, organisms will be morphologically different from their typical microscopic shape and importantly the natural immunogenic structures of organisms' components (e.g. LPS) would be denatured as a result of morphologic changes which usually leads to decreased immunogenicity.

According to chemical analysis of samples, optimized phenol extraction method is the method with the most LPS yield and concentration. As shown in Table 2, the highest concentration of LPS was measured in the extracted sample with optimized phenol method. The protein contamination of this sample is less than the sample extracted by phenol method (Westphal and Jann method). Since the application of LPS as an antigen is the main aim of extraction and in the other hand, proteins are highly immunogenic molecules, decreased content of protein makes the immunologic evaluation of the extracted LPS easier and more accurate. The purity of extracted LPS causes more specific immune response including both humoral and cell-mediated responses. The application of *Brucella* LPS in candidate vaccines and probable potency of this component to be used as an adjuvant are new studied and hot topics in vaccine technology. These new aspects of *Brucella* LPS application reveal the increasing importance of the extracted LPS samples purity. The KDO content in the optimized phenol extracted sample has been efficiently increased in comparison with other samples. KDO is unique structure and a derivative molecule of LPS and high concentration of this component in the extracted sample shows the fidelity of extraction method. Higher content of KDO in parallel with the increase of LPS concentration is a sign of optimization and verifies the sensitivity of our LPS concentration measurement assay. The optimized butanol extraction method leads to more LPS concentration and KDO content in comparison with the classic method (Morrison and Leive, 1978). Since the butanol method is a simple and suitable method for LPS extraction in the laboratory and would be applied in molecular and immunological studies, our optimized butanol extraction may be a better method for LPS extraction in the laboratory (but not in industrial scale). Present comparative study of LPS extraction could be compared to previous study published by some of this study research group members (Shapouri *et al*., 2007) but this new study is a novel project in some aspects: We have extracted LPS by four methods (versus three methods) and optimized both phenol and butanol extraction methods (versus optimization of phenol method).

CONCLUSION

Our present optimization in phenol extraction method includes more modifications than previous study. We have also assayed the LPS concentration and report the concentration of extracted samples measured by chromogenic LAL assay (versus yield of extraction expressed as percentage). Also we treated samples with DNase and RNase to reduce nucleic acid contamination in our optimized method (versus no enzymatic digestion) which caused less nucleic acid content in comparison to previous study. According to chemical analysis of the extracted sample we finally concluded that optimized phenol extraction is the best method with the least protein and nucleic acid contaminations and would be recommended to etract and purify *Brucella* LPS for industrial purposes.

REFERENCES

Alton, G.G., L.M. Jones, R.D. Angus and J.M. Verger, 1988. Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris.

Carmichael, L.E., 1990. *Brucella* canis. In: K. Nielsen and J.R. Duncan (Ed.). Animal brucellosis. CRC Press, Inc., Boca Raton, Fla., pp: 335-350.

Corbel, M.J., 1997. Brucellosis: An overview. Emerg. Infect. Dis., 3: 313-321.

Corbel, M.J. and W.J. Brinley-Morgan, 1984. Genus *Brucella* Meyer and Shaw 1920, 173 AL. In: N.R. Krieg and J.G. Holt (Ed.). Bergey's Manual of Systematic Bacteriology. The Williams and Wilkins Co., Baltimore, Md., 1: 377-388.

De Bagues, J.M.P., S. Dudal, J. Dornald and A. Gross, 2005. Cellular bioterrorism: How *Brucella* corrupt macrophage physiology to promote invasion and proliferation. Clin. Immunol., 114: 227-238.

Lapaque, N., I. Moriyon, E. Moreno and J.P. Gorvel, 2005. *Brucella* lipopolysaccharide acts as a virulence factor. Curr. Opin. Microbiol., 8: 60-66.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Morrison, D.C. and L. Leive, 1978. Fractions of Lipopolysaccharide from *E. coli* O111: B4 prepared by two extraction procedures. J. Biol. Chem., 250: 2911-2919 .

Shapouri, R., A. Mohabati Mobarez, H. Ahmadi, B. Tabaraie, R. Hosseini Doust, D. Norouzian, A. Zavaran Hosseini and S.D. Siadat, 2007. Optimization of *Brucella abortus* fermenter culture conditions and LPS extraction method for antigen production. Research Journal of Microbiology.

Texas A and M University Press, 2003. College Station. Bacterial Endotoxins Test, USP 26 NF 21.

Weissbach, A., 1959. The formation of 2-keto-3-heptonic acid in extracts of *E. coli* B. J. Biol. Chem., 1234: 705-709.

Westphal, O. and K. Jann, 1965. Bacterial lipopolysaccharides. Extraction with Phenol-water and Further Application of the Procedure In: R.L. Whistler, J.N. BeMiller and M.L. Wsolt'rom (Ed.). Methods in Carbohydrate Chemistry. Academic Press Inc., New York, 5: 83-91.

Wright, P.F. and K.H. Nielsen, 1990. Current and Future Serological Methods. In: L. G. Adams (Ed.). Advances in Brucellosis Research. Texas A and M University Press, College Station, pp: 305-320.