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Full Length Research Paper

A simple method for non phenolic extraction of lipopolysaccharide from *Salmonella typhimurium* and *Salmonella enteritidis* with high purity and pyrogenicity in rat

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Salmonella **species are the members of Enterobacteriaceae family, these organisms are gram- negative flagellated bacillus and most of their serotype except *Salmonella gallinarum* and *Salmonella pullorum* are motile. Lipopolysaccharide in gram-negative bacteria has the complex construction and is composed of three parts including the complex part named fat A, polysaccharide, and side chain O. There are different methods for extraction, separation and purification of LPS which are expensive or poisonous and less safe. In this study, the methanol-chloroform method is introduced because it saves time, expense and lack of usaging phenol. Lipopolysaccharide of *S. typhimurium* and *S. Enteritidis* were extracted using methanol-chloroform method. The extracts were compared with standard lipopolysaccharide via SDS-PAGE following silver staining. Pyrogenicity effect of *Salmonella* lipopolysaccharide was studied on 12 rats by using 100 and 150 µg/kg of LPS. The results showed that extracted bands out of lipopolysaccharide of both bacteria, is same as their standard. The LPS extract of *Salmonella typhimurium* revealed increasing of rat body temperatures (1 to 2°C) in both 100 and 150 µg/kg dosages after 2 h treatment. While the rat body temperature increases by the dose of 150 µg/kg, higher than the dose of 100 µg/kg. Considering the results, lipopolysaccharide bands extracted by using methanol-chloroform method and comparing them with standard samples could be a proper method with less expense, and much safety in comparison with the other methods and also it has a very high yield.**

Key word: *Salmonella,* lipopolysaccharide, SDS-PAGE.

INTRODUCTION

Salmonella species are the members of Enterobacteriaceae family, these organisms are gram-negative flagellated bacilli and all serotypes are motile, except *Salmonella gallinarum* and *Salmonella pullorum*. Salmonella spp are aerobic or facultative anaerobic and their optimum growth condition is at 37°C with pH=6-8 (Patrick et al., 2005; Pakzad et al., 2007; Moran, 1996). *Salmonella* species are classified based on Kauffman-white method in which more that 2300 serotype is derived according to the antigenic structure of lipopolysaccharide

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of the cell surface (AgO) and flagellate proteins (AgH) (Patrick et al., 2005). Lipopolysaccharide has a complex structure in gram-negative bacteria and is composed of three parts including, complex part named lipid A (the source of LPS is on the membrane and has immunologic property), polysaccharide (is composed of 10 to 15 sugar and has a special role in penetration membrane) and specific O side chain (O side chain composed of repetitive units of 7 sugar and has a special role in bacteria antigenicity). The LPS combination is the most surface part of the cell wall of gram-negative bacteria and is concerned as their endotoxin. This combination has heat resistance with molecular weight varied 3 to 5 kD to several millions which can be separated from membrane cell by Phenol aquatic solution (Prescott et al., 2002). This combination causes the serve reactions in immune system and highly poisonous for animals (called endotoxin) (Patrick et al., 2005; Pakzad et al., 2003; Rastegaret et al., 2008; Moran, 1996). Endotoxins of gram- negative bacteria are attached to cell wall of bacteria and cannot separate from it unless the bacteriolysis is done and the bacterium is decomposed. When LPS is degraded, the toxic part will be attached to lipid A. Polysaccharides are the formers of surface antigens of bacteria and are called Antigen O. LPS molecule is attached to outer membrane of cell wall by hydrophobic bands. The place of LPS synthesis is in the cytoplasm membrane and after synthesis is transferred to its final location whiles it is ready (Patrick et al., 2005).

LPS is one of the strong stimuli for immune responses. The B cells are activated and stimulated the other cells for releasing IL-1 and IL-6 and TNF and other factors (kim et al., 2007). The pure LPS can induce the strong reaction by itself. Phagocyte cells from the macrophage-monocyte group are important intermediates to these responses. Monocytes are very sensitive to LPS and can produce inflammatory protein products such as TNF-α1 and IL-1B at the very low level of LPS. These proteins have very important roles in defense of host against gram negative infections and also are concerned as key intermediate of septic shock (Russell et al., 1995). There are different methods for extraction, separation and purification of LPS which are extraction method with butanol (Morrison and Leive, 1975), ether (Ribi et al., 1961), Hot-Phenol (Westphal and Jann, 1965), proteinase K (Hitchcock and Brown, 1983) and EDTA (Goldman and Leive, 1965). Through these methods, Hot-Phenol is the most common one. Hot-Phenol method is used for extracting high amount of LPS commercially, regard to its cancerous and poisonous nature.

Phenol or carbolic acid was used for the first time in 1968 by Jozef lester, England scientist for disinfecting the operation room and the mechanism of its effect was denaturing proteins and lyses microorganism. Today the use of phenol has been decreased because of high price and poisoning and its derivations are more used, due to the problems of this compound, if liquid phenol was used, the steam hood should be used.

In this study the methanol-chloroform method was used to extract LPS because of its speed, low-expenses and lack of using phenol.

MATERIALS AND METHODS

Screening method

S. typhimurium and *S. enteritidis* were provided from Iranian reference health laboratory. Suspension cultures were transferred to the test tubes and the samples were centrifuged for 30 min in 2500 to 3000 rpm. Then, supernatant was transferred into a test tube. 2 ml of alcohol 95% was added to the test tube possessing sediment and vortexed till the bacteria sediment and alcohol were mixed properly and sediment was separated from the bottom of the test tube. After vortexing, the test tube was centrifuged for 10 min in 2000 rpm. The supernatant was discarded and sediment was washed with 2 ml alcohol and again vortexed. The procedure was repeated three times as explained previously. Finally, the sediment was mixed with alcohol and centrifuged for 10 min in 2000 rpm. The supernatant was discarded and sediment was dried by placing the tube under the hood till the rest of alcohol was evaporated. The dried bacterium was re-suspended by 1 ml EDTA 10% and then it was sonicated. 1 ml of saturated methanol/chloroform (1: 2 ratios) was added to bacterium-EDTA solution. The tube lid was covered by paraffin and shaked for 2 h following 10 min centrifuging in 2000 rpm. Three layers were formed; methanol, left biomass including cell lysate which was discarded and chloroform top to button respectively. Chloroform and methanol layers were separated and poured in new tubes and permit to evaporate completely. The dried pellet was LPS bacterium.

The LPS extract of *S. typhimurium* and *S. enteritidis* species along with their standard LPS were electrophoresed using 12% SDS-PAGE method (Madigan and Parker, 2001). Standard LPS belong to *S. enteritidis* and *S. typhimurium* were provided by Sigma Co. (L6011-100MG - 088K4014) and Pastor Institute in Tehran respectively. The gel was stained by silver staining methods based on Sambrook 2001 protocol.

In the electrophoresis method, the following ingredients are used to make 12% acryl amide gel from 20 ml acryl 30%, 5 ml TBE (10X), 600 µl AMS, 30 µl TEMED, 24.5 ml distilled water and in the SDS-PAGE method, the separative gel 12% is used containing 5.3 ml acryl amide 30 and 0.8% bisacrylamide, 6.5 ml water, 3.7 ml Tris-Cl/SDS 4X with pH=8.8, 2.88 gram urea, 50 µl APS 10% and 10 µl TEMED and separation process was done by the voltage 100 for 2 h.

Pyrogenicity test in rat

In this study, pyrogenicity test were examined by using 12 Wistar race rats which is provided by Pastor Institute. Two doses of 150 and 100 µg/kg lipopolysaccharide on 12 rats were injected in the form of intraperitoneally (i.p.) and after 2 h, the rectum temperature was recorded in the form of rectal as its results are shown in the Figures 1 and 2.

RESULTS

In the wells of 1 to 8 in Figure 3, the samples were loaded by 10 µl. Bands 1 and 2 are related to the extracted LPS of *Salmonella enteritidis*, bands 3 and 4 are related to standard LPS of *S. enteritidis* and band 5 and 6 related to standard LPS of *Salmonella typhimurium*, and bands 7 and 8 related to extracted LPS of *S.*

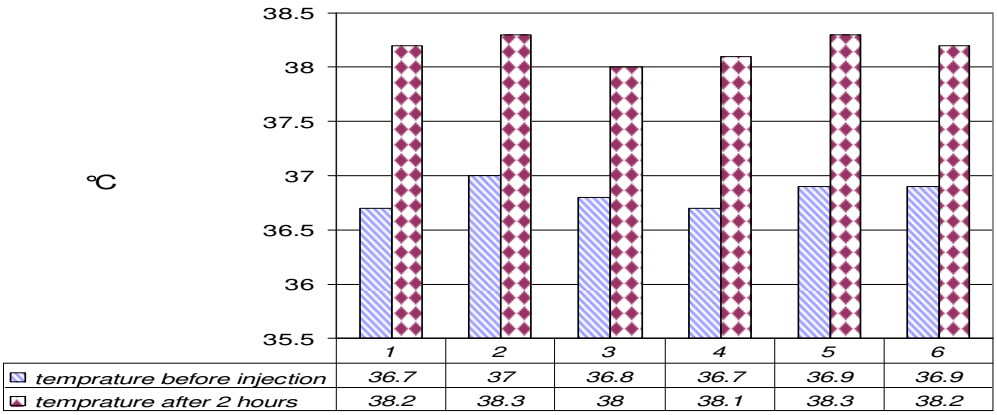

Figure 1. The measurement of increase the temperature from 1 to 2 centigrade in the form of rectal, 2 h after the injection of 150 µg/kg lipopolysaccharide in through intraperitoneally to albino Wistar rats.

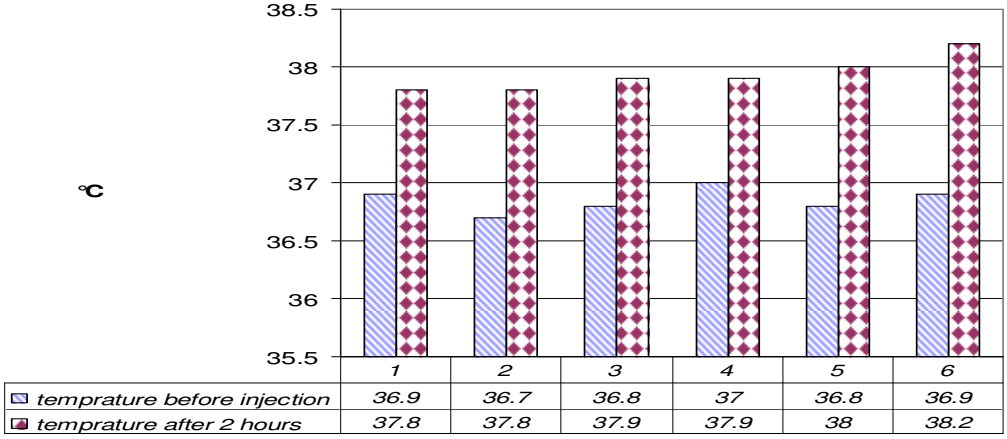

Figure 2. The measurement of increase the temperature from 1 to 2 centigrade in the form of rectal, 2 h after the injection of 100 µg/kg lipopolysaccharide in through intraperitoneally to albino Wistar rats.

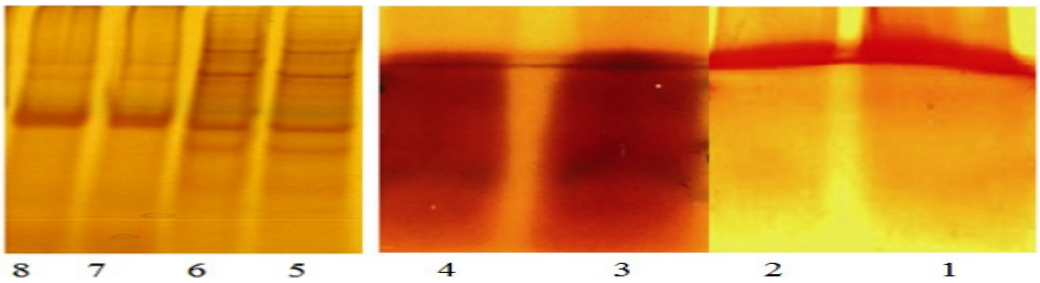

Figure 3. Bands 1 and 2 are related to the extracted LPS of *Salmonella enteritidis* (mutant rough pour H.g.m).bands 3 and 4 related to standard LPS *of Salmonella enteritidis* (Sigma L-6011-100 MG (for support of authenticity of extracted bands, and band 5 and 6 related to standard LPS of *Salmonella typhimurium*, and bands 7 and 8 related to extracted LPS of *Salmonella typhimurium*.

typhimurium.

LPS extracts with method which is introduced here showed high purity in comparison to standard LPS by running on SDS-PAGE (Figure 3). LPS extracts also showed the same molecular weight as standard LPS. This shows that the purification is done properly and this

method has an excellent and high efficacy.

The bands on the top of gel are showing the high molecular weigh which is in ladder shaped are related to LPS containing O-chain and the bands on the bottom of the gel is related to LPS lacks O-chain.

The result of pyrogenicity test showed that temperature increasing in the rat treated with *S. typhimurium* LPS in both doses of 100 and 150 µg/kg. It was also revealed higher body temperature with the dose of 150 µg/kg in rats studied.

DISCUSSION

Salmonella is one of the most important causes of food-born diseases in world. The most important serotypes isolated from human being are *S. typhimurium* and *S. enteritidis* (Soumet et al., 1999). Stain with silver nitrate was applied by Tsai-Frach for the first time (Tsai and Frasch, 1982), and this method is comparable with auto-radiography and gives the same results (Hitchcock and Brown, 1983; Tsai et al., 1983). By using these methods, they can get S-form lipopolysaccharide contains one heterogeneous which is mixture of LPS molecules with different amount of repeating oligosaccharide from 0 to 40 (1 Palva and Makela, 1980: 8; Tsai and Frasch, 1982) Polysaccharide part of lipopolysaccharide molecule is the reactivated part stained with silver nitrate (Golding et al., 1992). The present hexoses oxidization, make aldehyde groups which are able to act with silver nitrate. Fumsgard and et al. showed that classic method cannot stain the special strain of LPS including a low amount of fatty acid which washed in the very first fixation levels.

Therefore, they designed a modified silver staining technique by removing the fixation level and increase of the time of lipopolysaccharide alternative acidic oxidization from 5 to 20 min till the ability of showing all of the lipopolysaccharide is returned (Li and Parwinder. 2002).

In the studies of Golding et al. (1992) it was shown that poisoning of *Brucella abortus* LPS n deadly experiments in mouse and pyrogenicity in rabbit is much less than the LPS of E-coli, but the number of endotoxin units of 1 mg *Brucella abortus* LPS is more than 1 mg of LPS in Ecoli (Pakzad et al., 2003) on the other hand, intraperitoneal injection of LPS in rats (400 to 800 µg/kg, i.p.) was shown to increase the concentrations of norepinephrine, dopamine, serotonin and their metabolites in the hypothalamic paraventricular nucleus measured by HPLC 5 h after the treatment (Mohan et al., 1999; Ken, 2001. Peggy (1993) suggested that, high doses of rat injected with LPS exhibited an increase in colonic temperature which is in line with the results of this study. Sirakarn et al. (2010) suggested, lipopolysaccharide (LPS; 50 µg/kg) injected intra-muscularly to male Wistar rats. Rectal temperature was measured at 1 h intervals for 7 h after the extract/ drug administration. The rectal temperature of normal rats (normothermic rats) was also measured at 1 h intervals for 7 h. The maximum increase in rectal temperature was reached at 4 h (1.84°C) giving the maximum mean rectal temperature of 38.44°C after which there was a decrease. The maximum mean rectal temperature produced by LPS in the presence of 50, 100, 200 and 400 mg/kg of the EFR were 36.99, 36.94, 36.89 and 37.04°C, respectively (Sirakarn et al., 2010). Soszynski and Chelminiak (2007) suggested, lipopolysaccharide (LPS; 50 µg/kg) injected to rats, had increase in rectal temperature (37.94 and 37.79°C) at 120 and 300 min post injection. Study by Benamar et al. (2000) suggested that, LPS injected i.p. (50 µg/kg) induced an increase in temperature body that peaked at 180 min (1.25°C) and again at 5 h it was (1.52°C) and these results are in line with the results of the present study. Because of importance of LPS in pathogenesis of illnesses related to digestion system, day by day its importance for recognition and treatment is increased, it is expected that by change in the present methods, it can be seen as the simple, fast and cheep method as it was applicable in this study (methanol-chloroform method).

Conclusion

In applying the electrophoresis process. SDS-PAGE. And studying the composed bands for surveying the extracted LPS from the *S. typhimurium* and standard LPS and comparing it with extracted lipopolysaccharide out of *S. enteritidis* and its standard LPS, it is concluded that the extraction of LPS through the methanol-chloroform method is the proper method which is also accompanied by low expenses and high yield, we can properly come to this conclusion by the aforementioned picture. Also, by using the SDS-PAGE method, extracted LPS bands are compared with its standards and it is observed that the extraction processes did well and the same bands are produced.

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