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Physiological and Immunological Effect of Lipopolysaccharide of *Escherichia coli* was Extracted by Hot Phenol-Water in Rabbits.

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ABSTRACT

In this study lipopolysaccharide of Escherichia coli was extracted by hot phenol-water method as described previously with some modifications ,the bacteria was isolated from urinary tract infection . The endogenous pyrogen activity of extracted LPS was evaluated resulted in substantial increase of rabbits' body temperature (mean: 1.6°C).the effect of extracted LPS on immune parameters was studying in rabbits contain three rabbits in each group ,were injected Intramuscule (I.M.) Intravein(I.V.), I.M. & I.V. to give mean total protein 54.72,54.97,59.1 in Primary immune response,60.67,62.6,66.43 in secondary immune response respectivily and mean of C.R.P.in secondary immune response revealed inceasing more than mean in Primary immune response.

Keywords: *Escherichia coli,* phenol-water, rabbit, immunology.

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INTRODUCTION

Urinary tract infection (UTI) is one of the most spreading infectious diseases, having an estimated number of 150 million cases in the world (Bashir et al.,2008). The main cause of UTI is Gram-negative bacteria , especially Escherichia coli which considered the most prevalent causative agent because it is responsible for more than 90% cases of UTI (Behzadi & Behzadi,2008). Bacteria E. coli contain lipopolysaccharide (LPS) as a main components of their cell wall like all other gram negative bacteria(Kong et al.,2011). LPS is an immunodominant molecule that is important for the virulence and pathogenesis of many bacterial species, including Pseudomonas aeruginosa, Salmonella species, and Escherichia coli (Jann,1987; Pier ,2007).

The outer membrane of gram negative bacteria is an asymmetric bilayer, composed of phospholipids in the inner leaflet and lipopolysaccharide in the outer leaflet. LPS is structurally composed of three parts: a hydrophobic lipid A that anchors the LPS molecule to the bacterial outer membrane, a phosphorylated core oligosaccharide that comprises ~10 sugar residues (Amor et al.,2000) and a repeating oligosaccharide unit referred to as the O-antigen polysaccharide (Stenutz et al.,2006). The core can be subdivided into a conserved inner core that contains 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and L-glycero-D-manno-heptose (Hep) and a less conserved outer core that consists of hexose residues. The LPS layer acts as a permeability barrier for small molecules; thus, essential nutrients and ions can only access the cell through barrel porins that are embedded in the outer membrane (Nikaido,2003).

Hydrophobic lipid A is highly preserved and mainly responsible for the toxicity of the molecule, while the O-antigen is repeating hydrophilic oligosaccharide side chain that is specific to bacterial serotype (Erridge et al.,2002). Lipopolysaccharide is a common virulence factor and binds to toll-like receptor 4 (TLR4), subsequently triggers the production of proinflammatory cytokines, initiating the innate response against uropathogenic E. coli (UPEC) (Rudick et al.,2010). decrease endotoxic activity of recombinant proteins purified from *E. coli* BL21 backgrounds and a feasible approach to modify lipid A structure for alternative purposes such as mono-phosphoryl lipid A (MPL) as vaccine adjuvants (Liu et al.,2015) . The aim of the study was to extract LPS from E. coli by hot phenol-water method and to benefit from this study to understand the pathogenesis of UTI caused by E. coli to study the role of LPS in this infection and to study immunological parameters by studying the possibillity of use LPS extracted from E. coli as antigen to induce immunity.

Sample Collection

A total of 150 samples were collected from HillaTeaching Hospital,Babil,Iraq, with clinically diagnosed UTI. The diagnosis of UTI was based on microscopic findings of more than 5 white blood cells per high power field (1000× for high power) and a colony count of 105 colony forming units/mL of a single pathogen. Patients with UTI were selected randomly between ages 6 month to75 years, comprising of both male and female, in a period from April -December 2015. We took the patients who were not taking antibiotic for treatment. The urine of 150 patients was sampled by clean catch of midstream urine. Urine samples were delivered to the laboratory within 1 hour of collection and processed within 2 - 4 hours. 82 samples have presented E. coli upon isolation and identification as UTI causing organism, only one E. coli isolate was selected for LPS extraction

Identification of bacterial pathogens

The bacterial isolates were identified and confirmed using standard microbiological method which included Gram staining, colonial morphology on media(on blood agar, MacConkey s agar) , and growth on selective media, lactose fermentation, catalase, oxidase, coagulase, indole, citrateutilization, urease tests and hanging drop preparation for motility (Collee et al., 1996).

LPS extraction

LPS was extracted by hot phenol-water method as described previously with some
modifications (Westphal & Jann,1965; Rezania et al.,2011). In brief, bacterial suspensions (108 colony-forming units/mL) were centrifuged at 10,000×g for 5 min. The pellets were washed twice in PBS (pH=7.2) (0.15 M) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Pellets were then resuspended in 10 ml PBS and sonicated for 10 min on ice.

At the next step, an equal volume of hot (65-70°C) 90% phenol was added to the mixtures followed by vigorous shaking at 65-70°C for 15 min. Suspensions were then cooled on ice, transferred to 1.5 mL polypro pylene tubes and centrifuged at 8500×g for 15 min. Supernatants were transferred to 15 mL conical centrifuge tubes and phenol phases were re-extracted by 300 µL distilled water. Sodium acetate at 0.5 M final concentration and 10 volumes of 95% ethanol were added to the extracts and samples were stored at -20°C over night in order to precipitate LPS. Tubes were then centrifuged at 2000×g 4°C for 10 min and the pellets were resuspended in 1 ml distilled water. Extensive dialysis against double distilled water at 4°C was carried out at the next step until the residual phenol in the aqueous phases was totally eliminated. Final extracted LPS product was lyophilized and stored at 4°C.

Rabbits:

In this study we use 15 local rabbits(Oryctologus cuniculus) males12 were used in immunological experiment while the other 3 rabbits were used in pyrogenicity test ,their age between (8-9) months and weighing between (2-3.75) kg were kept in animal house of biology department,Babylon university .

Rabbit pyrogen test

Pyrogenicity was tested in an air conditioned room. Rabbits were given an intravenous injection of 5 ng/kg of body weight of extracted LPS. Rectal temperatures were measured with indwelling rectal thermostats and recorded before injection and 4 hr after pyrogen administration. One rabbit was injected with sterile PBS as control.(Rezania et al.,2011).

Total protein : was evaluated according to Biuret method (Bishop *et al.*, 1985).
C.R.P.: were evaluated in accordance with manufacturing company.

RESULTS

E. coli was isolated from UTI in table (1) and it was the first causative agent.

Table(1)types of bacterial isolates from UTI patients

Bacterial type	Samples numbers	Samples percentage
Escherichia coli	82	54.6%
Other bacteria	68	45.3%
total	150	100%

In table (2) we see the results of increasing rabbits's temperatures after Injection of LPS.

Table(2)Evaluation of extracted LPS pyrogen activity by rabbit pyrogen test

rabbits	Initial rectal temperatures	Injection of LPS
1	37	38
2	36.7	39
3	37	38.7
mean	36.9	38.5
Control rabbit which received PBS	37	37

Table (3) is showing thedifference between rabbits's weight in primary immune response which detected after one week of immunization and in secondary immune response which detected after two weeks of immunization.

Table (3)the effect of extracted LPS on the weight of injected and control rabbits.

	rabbits	primary immune response.	secondary immune response.
Intramuscule(I.M.)	1	3.5	2.5
	2	3	2
	3	3.5	2.5
mean		3.33	2.33
Intravein(I.V.)	1	3.5	3
	2	3	2.75
	3	3.75	2.5
mean		3.41	2.75
I.M. & I.V.	1	3.5	3
	2	2	2
	3	3.5	2.75
mean		3	2.58

The effect of extracted LPS on protein is evaluated in table(4)

Table(4)total protein in serum of rabbits immunized by extracted LPS.

	rabbits	Primary immune response.	secondary immune response.
Intramuscule(I.M.)	1	48.2	55.8
	2	62.28	68.93
	3	53.7	57.3
mean		54.72	60.67
Intravein(I.V.)	1	54.72	62.9
	2	49.99	57.8
	3	60.2	67.2
mean		54.97	62.6
I.M. & I.V.	1	68.2	68.8
	2	57.3	70.2
	3	51.8	60.3
mean		59.1	66.43

Table(5)C.R.P.in primmary immune response.

	rabbits	titer	concentration
Intramuscule(I.M.)	1	2	12
	2	4	24
	3	2	12
mean		2.66	16
Intravein(I.V.)	1	8	48
	2	8	48
	3	4	24
mean		6.66	40
I.M. & I.V.	1	4	24
	2	8	48
	3	4	24
mean		5.33	32

Table(6)C.R.P.in secondary immune response.

	rabbits	titer	concentration
Intramuscule(I.M.)	1	8	48
	2	4	24
	3	2	12
mean		4.6	28
Intravein(I.V.)	1	16	69
	2	8	48

	3	8	48
mean		10.6	55
I.M. & I.V.	1	8	48
	2	8	48
	3	8	48
mean		8	48

DISCUSSION

We have described a method of extraction LPS away from other cellular components to study its characters from bacteria E. coli which was isolated from UTI patients .in this study the E. coli the main causative aget with ratio 54.6% this agree with(John et al.,2015). This method of extraction provided high-quality LPS(Rezania et al.,2011) that can be useful in a number of different visualization methods such as in LPS pyrogen activity and in immunological parameters.

Many studies had been done to extract LPS from E.coli (Westphal & Jann ,1965; Salati et al.,1987; AL-Harbi & Austin,1992; Rezania et al.,2011). we use method hot phenol-water with modification such as others (Chowdhury et al.,2015). Rabbit pyrogen test was done to evaluate the functional activity of LPS and here we used in vivo assay to detect this activity and we can see that increasing of rabbits' body temperature this agree with(Rezania et al.,2011). the LPS extracted from bacteria E.coli by this method effect on rabbits' wheight by decreasing their wheights this refers to LPS ability to induce infection in animal models(Opdal et al.1993) .the raise of total protein in the sera of immunized rabbits in compare with non immunized rabbits proved that LPS extracted from bacteria E.coli stimulate immune response because of the potency immunization of LPS (Yahya,2001).

There are differ in CRP concentration in primmary and secondary immune response in immunized rabbits with LPS this differences is caused by increasing the circulation proteins in plasma (Rastaldi et al,2000).as well as there are different models for secreting the CRP in blood depending on the kind of antigen that induce the humoral and systemic immune response which specific for antigen(Lynch,2006).

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