The protective role of lipopoylsaccharide against Avian pathogenic *E.coli* in broilers

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Abstract

The present study was designed for the development of a vaccine composed of different antigens (LPS, OMP, LPS+OMP and Bacterin) of avian pathogenic *E. coli* O78 to protect of chicken against colibacillosis. A total of 100 clinical samples from ill broiler chicks with colibacillosis were collected from different regions of AL-Qadisiya province. All subjected to isolation, identification, biotyping, serotyping and antimicrobial agent sensitivity for APEC by using biochemical, Api – 20E, and biotyping using patterns of different fermentation and sertyping by monospecific antisera for O and H antigen. The study showed that the prevalence of resistant APEC O78 isolates is very high. Of a battery of antibiotics used, these isolates were sensitive for only few antibiotics; Colistin sulphate and Gentamycin. The study have carried out in two stages; first stage included cultivation of identified isolates, extraction and purification of target antigens, and analysis for carbohydrate and protein contents. In addition, gel filtration and SDS-PAGE were performed for extraction of antigens; i.e. LPS. Second stage included the field trial, where 300 of 1 day aged broiler chicks were divided into 6 equal groups. Four of five challenged groups (inoculated with $1x10^9$ cells/ml) were immunized with LPS (2.5 mcg/bird, sc) and OMP (2 mcg/bird, sc). The first initial dose was given at 5th day old of broiler chicks followed by a booster dose after 9 days. A clinical follow-up, morbidity and mortality were monitored daily. The challenge dose was given at 29 days old for all of studied groups. At 33rd day old, histopathological examination of different vital organs, CFU/g, cytokins assay for selected interleukins (IL-1B, IL-6, and TNF-α;TLiA) and immunoglobulines (IgG and IgM) assessment were performed. Mortality rate was significantly lower than that of positive control (56.7%) after bacterial challenge. Significant difference in IgG concentration was shown among groups. It was higher in vaccinated groups compared with positive control. Serum IL-6, IL-1B, and TNF-α concentrations were significantly higher in the 1st vaccinated group and placebo controls compared with positive group. Infected chicks showed gross lesions represented by airsaculitis, pericarditis and hepatitis, whereas, vaccinated groups appeared no significant gross lesions in air sacs, heart and liver. It is concluded that LPS and OMP of avian pathogenic *E. coli* O78 can protect chicken against colibacillosis.

Introduction

Colibacillosis which is caused by *Escherichia coli* (*E. coli*) causes considerable economic and welfare problems in broilers (1,2). Due to its frequent occurrence and its adverse effects on growth and health, clinical disease consists of respiratory signs, growth retardation, reduced feed intake and increased mortality (3). Good hygiene, vaccination and therapeutic treatment do not provide sufficient protection against colibacillosis. Vaccination against *E. coli* is used in the primary agents that provoke secondary colibacillosis may themselves act as primary agents. Therapeutic treatment is expensive (Turtare *et al*., 1990, Otaki, 1995; Vandemaele *et al*., 2002b; Vandekerchove *et al*., 2004). The potential of controlling *Escherichia coli* infections in commercial poultry through vaccination has been explored widely over the past several decades. In these attempts live or killed bacteria and their various cellular components have been used to immunize chickens (Deb and Harry, 1976; Nagaraja *et al*., 1984; Gyimah & Panigrahy, 1985; Melamed *et al*., 1991; Frommer *et al*., 1994; Kariyawasam *et al*., 2002; Chansiripornchai, 2009). A number of experimental vaccines have been developed for the prevention of colibacillosis in poultry, vaccination with *E. coli* bacterins (Deb and Harry, 1976; 1978; Cessi, 1979; Melamed *et al*., 1991), or a multivalent pilus vaccine (Gyimah *et al*., 1989) by subcutaneous injection provided protection against challenge with homologous *E. coli* strains. (Deb and Harry, 1976).

Materials and methods

Collection of samples, and identification and isolation of *E. coli*.

A – Clinical observations: In most cases, broiler chicks were listless with ruffled feathers and indications of fever, labored breathing, occasional coughing and rales. Bacteria were recovered originally from a variety of tissues, with most isolates being collected from air sac, pericardium, heart, liver, gall bladder and spleen.

B – Samples: A total of 100 specimens were collected randomly from broiler chicks with colibacillosis, during the period extended from May, 2010 to December, 2010. Collection was carried out under aseptic technique with care must be take to avoid faecal contamination of samples, the swabs collected from air sac, liver, heart, gall bladder and spleen after that placed in 2ml of transport media (Brian–heart infusion broth), immediately and following collection the samples were transported to laboratory by cool box.

Specimens were streaked onto Blood agar plates and MacConkey agar and Incubated at 37 C^{o} for 24 hrs. Diagnosis depend on morphological character (shape, color and size) of colony. Typical lactose ferment colonies were picked randomly and transferred to MacConkey agar plates and Eosin methylene blue agar plates and incubated for additionally over night, then

the colonies were individually identified using gram stain and traditional biochemical tests according to Collee *et al*. (1996).

C– Gram stain: single colony from Eosin methylene blue agar by loop was spread on clean slide, fixed and stainend with gram stain according to (Quinn *et al*., 1998), then examined under oil immersion.

D– Culture on MacConkey agar: loopful of the suspected culture was streaked on the prepared MacConkey agar and incubated at 37 Cº for 24 hrs. Avian pathogenic *E. coli* are lactose fermenter (pink colony).

E– Culture on Eosin methylene blue agar: loopful of the suspected culture was streaked on the prepared Eosin methylene blue agar and incubated at 37 Cº for 24 hrs. Avian pathogenic *E. coli* colonies appear greenish metallic sheen.

F– Biochemical tests:

1 – Oxidase test: A strip of filter paper which soaked previously with oxidase reagent (1 % solution of tetramethyl–p–phenylene–diamine dihydro chloride), then the colony was picked up with a sterile wooden stick and smeared over the filler paper. A positive result was indicated by an intense dark purple during 5 – 10 seconds (MacFaddin, 2000).

2 – Catalase: It done by spreading single colony of bacteria from *E. coli* on a clean slide, then mixed with a drop of hydrogen peroxide (3%). The production of O_2 bubbles as a positive reaction (Quinn *et al*., 1998).

3 – Urease test: It was made by culturing the isolate on slant of urea agar and incubated at 37 Cº for 24 hrs, and if color of media converging from yellow to pink color, indicate as a positive reaction (Collee *et al*., 1996).

4 – Indol test: This test is used to detect indol rings from the destruction of the amino acid tryptophan and carried out by addition of Kovac's reagent into 5 ml of peptone water culture placed in tubes and mixed gently , and the red ring was appeared as appositive reaction (Macfaddin, 2000).

5 – Methyl red test: This test was carried out by inoculating 4 ml of MR–VP media with 0.5 ml of bacterial suspension and incubated at 37Cº for 24 hrs, then 3 drops of MR added to the media and mixed gently. The development of clear red color is indicative of positive reaction (Quinn *et al*., 1998).

6 – Voges – Proskaur Test: MR-VP broth was inoculated with 0.5 ml of bacterial suspension and incubated at 37 Cº for 24 hrs. One ml of 40% KOH and 3 ml of 95% solution of α–naphthol were added. A positive reaction was indicated by the development of a pink color in 2–5 min. (Macfaddin, 2000).

7 – Citrate Utilization test: Simmon's citrate slant was inoculated with bacterial colony and incubated at 37Cº for 24 hrs. A blue color of media is an indication of positive reaction (Quinn *et al*., 1998).

8 – Gelatin liquefaction test: Bacterial colonies were cultured on gelatin media and incubated at 37Cº for 24 hrs. Non solidifying of gelatin after stored at 4º C for 10 minutes indicated positive reaction (Macfaddin, 2000).

9 – Triple sugar iron (TSI) test: The bacterial colony was grew on slant of TSI agar by stripping and streaking, then incubated at 37Cº for 24 hrs. Gas bubbles was appeared as indication of CO_2 formation, and black precipitate was appeared as indication of H_2S production (Quinn *et al*., 1998).

10 – Phenylalanine deaminase test: Slant of phenylalanine agar inoculated and incubated at 37 Cº for 24 hrs, then 4 -5 drops of ferric chloride reagent were added and mixed in order to cover the colonies with reagent. Green color was appeared as a positive reaction (Macfaddin, 2000).

11-Api–20E system (Analytical profile index for Enterobacteriaece test): According to Atlas (1995), this test (Api–20E system, BioMerieux, France) was used for diagnosis the bacterial isolates. This test have 25 strips, each strip contain 20 microtubes with contained dried material. The results were converted into numerical profile and compared with the company index. Each strip divided into seven parts, each part have three test numbered with (1, 2, 3) and each positive test is given specific number, but negative test not given any number, then all three number of each group which about 0–7 (7 digit profile), therefore finally we had seven number which compared with index to give genus and species of bacterial isolates.

Serotping of Avian pathogenic *E. coli* by agglutination test.

The serotyping of APEC isolates were performed by kit for veterinary laboratories Agency, UK. Surrey K Tis 3NB, UK for *E. coli* O2: K1 typing serum OK "F21" (Avian strain), *E. coli* O1: K1 typing serum OK "F1" (Avian strain) and *E. coli* O78: K80 typing serum OK "F103" (Avian strain). All are manufactured by VLA, UK. After diagnosis of bacterial isolates by gram stain, biochemical test and Api –20E system, the serotyping was performed.

Preservation of bacterial isolates:

The tentative isolates were cultured on sterile brain heart infusion broth and glycerol 20% and incubated at 37 C for 24 hrs, after turbidity appeared, they stored in a deep freezing (Bopp *et al*., 2005).

Fermentation of carbohydrates by *E. coli* isolates:

The *E. coli* isolates were characterized by their ability of utilize the following sugars: maltose, lactose, sucrose, dulcitol, adonitol, salicin, raffinose, dextrin, xylose, rhamnose and mannitol. The indicator used for sugar fermentation (Bromothymol blue broth) base was prepared by dissolving peptone (10g), sodium chloride (5g) and bromothymol blue (0.018 g) in 1 litter of distilled water. Each carbohydrate solution was prepared by dissolving 1% of corresponding sugar in the broth base medium, and only salicin was prepared at 0.5%. Each *E. coli* isolate was inoculated into prepared sugar medium and incubated at 37 C for 24 hrs. The test was recorded as positive when the medium turned from bluish colour to yellow (Gross, 1994; Bopp *et al*., 2005).

Antimicrobial drug sensitivity test:

In vitro antimicrobial drug sensitivity test was performed on confirmed *E. coli* isolates presenting as greenish metallic sheen colonies on Eosin methylene blue agar. The antimicrobial sensitivity test was proceeded according to Bauer *et al.* (1996). The Mueller Hinton agar used in antimicrobial testing. The selection of antibiotic disk concentration and interpretation of the zone size were done as recommended by the manufacturers (Oxoid, UK) and the National Committee for Clinical Laboratory Standards (NCCLS, 1990). The following antibiotics concentration per disk were used: ciprofloxacin (5 mcg), sulphamethazole–trimethoprim (25 mcg), tetracycline (30 mcg), chloramphenicol (30 mcg), ampicillin (10), amoxicillin (25 mcg), cephalothin (30mcg), cefotaxine(30 mcg), streptomycin (10 mcg), Gentamycin (10 mcg) and Colisitn (10 mcg).

LPS extraction and purification:

A) LPS Extraction: (Westphal *et al*., 1952).

Fifty hundred ml of brain heart infusion broth was inoculated by avian pathogenic *E. coli* and incubated at 37 Cº for 24 hrs. Bacterial suspension was examined with gram stain, then 100 Petri dishes of sterile brain heart infusion agar were prepared and inoculated with suspension of avian pathogenic *E. coli* (5 ml to each Petri dish) and incubated at 37 Cº for 24 hrs. Bacterial growth was harvested with sterile PBS (pH=7.2), then centrifuged by cooled centrifuge, 3000 rpm for 30 minutes. Thereafter, cells were washed three times with PBS (pH=7.2) and centrifuged by cooled centrifuge 2000 rpm for 10 minutes. Bacterial cells were suspended with formalinized PBS (PH=7.2) (solution no. 2) at 4 Cº for 18 hrs, centrifuged by cooling centrifuge 3000 rpm for 30 minutes, then washed three times with the same solution and the precipitate was collected and frozen.

B) Bacterial lyses by enzymes.

Bacterial cells were suspended with solution no. 5 (1:10 v/v) and mixed well on magnetic stirrer with high speed for 1 minute, then 0.1 g of lysozyme/ 5 g of bacterial cells added and the suspension was put on magnetic stirrer at 4 Cº for 18 hrs. Thereafter, suspension was incubated at 37 Cº for 20 minute, then the suspension was diluted by adding equal volume of solution no. 7. DNase and RNase solutions (l mcg/ l ml) were added, and the suspension was incubated at 37 Cº for 10 minutes, thereafter incubated at 60 ºC for 10 minutes.

C) Extraction with hot phenol: (Westphal *et al*., 1952).

The suspension (final step) was heated to 70 ºC in water bath, an equal volume from solution no. 6 at 70 ºC was added and the mixture was put on hot magnetic stirrer (70 ºC for 15 minutes).The mixture was put directly in iced water bath, then the mixture was centrifuged at 10000 rpm for 30 minutes at 7 ºC. Four layers formed from up to down: watery layer, intermediate layer, phenol layer and precipitate layer. Phenol and watery layers were drawn with Pasteur pipette each alone. Adding 3 volume of distal water to the two residual layers and centrifuged with the same speed and time. Watery layer was transported into dialysis tubes and dialysed against distilled water for 96 hrs until the phenol odour disappeared. The resulted sample from dialysis were transported to rotary evaporator to obtain a concentrated crude LPS solution.

D) Purification of LPS by Gel filtration chromatography:

A sapharose 6B, 75×1.5 cm, column was prepared and packed according to the instructions of the manufacturing company (pharmacia, Sweden).

Experimental birds and vaccination experiment:

Six hundreds unvaccinated broiler chicks (Ross) of mixed sex (aged 1d.) were obtained from a commercial Babel Hatchery in Adiwania province, and randomly divided into six groups (100 per each). All groups were housed together in the suitable hall, the concrete floor was covered with wood shavings, the temperature and relative humidity accorded with the requirements of broiler chicks. Commercial pullet feed and clean water were available *ad libitum* and staff decontaminated their footwear and put on clean hall coat. Birds were vaccinated as follow:

Group I (LPS group; T1): inoculated with LPS as 2.5 mcg/ g of broiler chick (0.2 ml/ chick, sc) on 5th day, then repeated after 9 days.

Group V (negative control group; C-): inoculated with PBS (0.2 ml/ chick, sc) on 5th day, then repeated after 9 days.

Group VI (positive control group; C+): inoculated with APEC (O78), were made using McFarland's tube and spectrophotometer to justify the challenge does (0.2 ml/ broiler chick, sc).

Blood samples were collected from five broiler chicks of each group to separate serum for immunological assay. Body weights were measured on 5, 20, 30, and 35 days of age. Feed and water consumption for each group between 1- 35 days should be measured. Mortality was defined as the number of broiler chicks that had died before the end of the study. Morbidity of the number of broiler chicks sick with or without lesion in either air sac, pericardium or perihepatic should be estimated.

Immunological assessment (ELISA):

Qualitative detection of chicken IgM and IgG classes antibodies, and IL-1β, IL-6, and TNF-α have been performed according to the manufacturer instructions (ABO, Switzerland).

c) Microscopic examination: Specimens (1 cm^3) were taken from liver, spleen, lung, bursa of fabricia and air sacs. The tissues were kept immediately in 10 % formalin buffer. After 72 hrs of the fixation the specimens were washed with tap water and then processing was routinely done with asset of up grading alcohols concentration from 70 % to absolute 100% for two hrs in each concentration to remove water from the tissue, then clearance was done by xylol. Thereafter, specimens were embedded with paraffin wax at 58 C on two stages, then blocks of specimens were made with paraffin wax and sectioned by rotary microtome at 5 mm for all tissue. All tissue were stained with hematoxylin and eosin stain and the histopathological changes were observed under light microscope (Luna, 1968).

Statistical analysis:

Data were translated into a computerized database structure. An expert statistical advice was sought for statistical analyses where computer assisted using SPSS version 13 (Statistical Package for Social Sciences). Frequency distribution for selected variables was done first. Compliance of quantitative random variables with Gaussian curve was analyzed using the Kolmogorov Smirnov test. All the outcome quantitative variables were non-normally distributed. Such variables are described by median and interquartile range.

Results

Incidence of collibacillosis:

Based on clinical manifestations and postmortem examination, the overall infection rate in the epidemiological study was 65%. A total of 100 chicks were examined for evidence of infection, the infection rate ranged between 50% in Hamza district to 80% in Afak district.

The differences observed in infection rate by source of chicks were statistically not significant (table 4-1).

Table (4-1): The infection rate by source of chicks in the prevalence study.

	Total	Positive evidence for infection	
Source of chicks	**N**	**N**	**%**
AL–diwanyia	20	11	55
Afak	20	16	80
Dagara	20	15	75
Shmeyia	20	13	65
Hamaza	20	10	50
Total	**100**	**65**	**65**

P (Chi-square) = 0.22[NS]

Results of biochemical test :

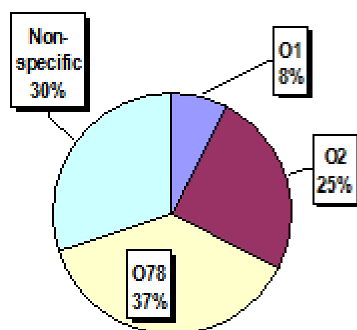
E. coli strain was diagnosed and the results were elucidated in appendix (1).

Definitive diagnosis:

Api–20E test Confirmed the diagnosis of *E. coli* isolates.

Serotyping:

Serotyping was performed on 40 cases with diagnosed infection. Serotype O78 was the most frequent (37.5%) followed by serotype O2 (25%) and O1 (7.5%). About a third (30%) had no-specific serotype (non-typable), table (4-4) and figure (4-3)



Figure(4-4): Pie chart showing the frequency distribution of cases with infection (n=40) by serotype.

Antimicrobial drugs sensitivity test:

As shown in table (4-5) and figure (4-4), Colistin sulphate was the antibiotic with highest sensitivity rate (93.8%) for culture positive specimens, followed by Gentamycin (84.6%).

Antibiotics with least reported bacterial sensitivity (9.2%) were Ampicillin, Amoxicillin, Cephalexin and Sulphamethazole–trimethoprim.

Table (4-1): Antibiotic sensitivity of cultured specimens (n=65).

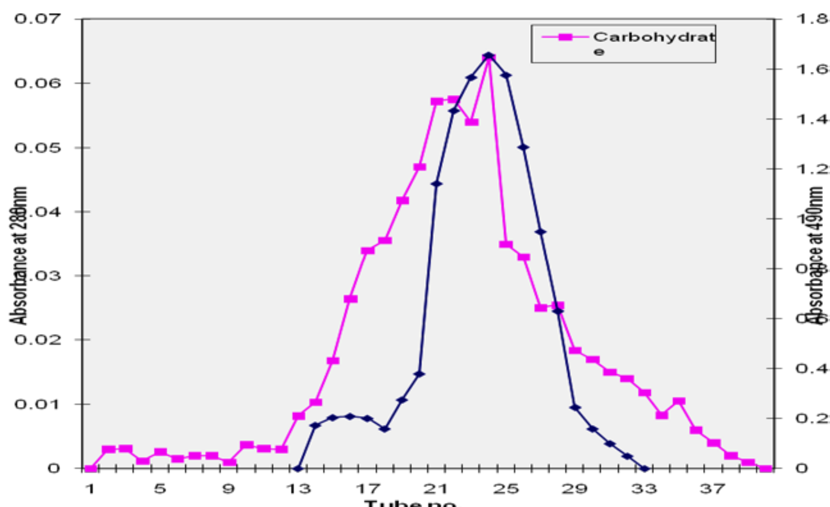
Antibiotic sensitivity (n=65)	N sensitive for specific antibiotic	Percent sensitive (%)
Colistin sulphate	61	93.8
Gentamycin	55	84.6
Ciprofloxacin	39	60
Streptomycin	36	55.4
Tetracycline	15	23.1
Cefotaxime	15	23.1
Chloramphenicol	15	23.1
Ampicillin	6	9.2
Amoxicillin	6	9.2
Cephalexin	6	9.2
Sulphamethazole–trimethoprim	6	9.2

Avian pathogenic *E. coli* (O78); Lipopolysaccharide:

LPS Extraction: Two hundred µg of crude LPS from APEC O78 were extracted from 20 g wet weight of bacterial cells.

LPS Purification: After the addition of 2 ml of blue dextran 2000 solution into the sepharose 6B column and measurement of absorbance value for each fraction at 280 nm ware length, the column void volume (vo) was determined by estimating the total volume of the fractions as determined from the start point movement of the blue dextran to the point that revealed the climax absorbancy of the blue dextran at 600 nm. Figure (4-6) shows that one peak had highest values of optical density of CHO and protein therefore peak one was represented as purified LPS of *E. coli* O78.

LPS content



Fraction number

Figure (4 -6) : Values of LPS constituents of APEC (O78)

Table (4-8): mortality rate among the 6 study groups for the period after vaccination until after bacterial challenge.

	First 5 days before vaccination			After the 1st vaccine dose			After the 2nd vaccine dose			After challenge with bacteria		
	Fatality			Fatality			Fatality			Fatality		
Groups	**Total at risk**	**N**	**rate (%)**	**Total at risk**	**N**	**rate (%)**	**Total at risk**	**N**	**rate (%)**	**Total at risk**	**N**	**rate (%)**
Control (not inoculated, PBL vehicle vaccine)	100	8	8	92	2	2.2	90	1	1.1	89	5	5.6
Positive control (inoculated but unvaccinated)	100	10	10	90	0	0	90	0	0	90	51	56.7
LPS vaccine	100	8	8	92	3	3.3	89	2	2.2	87	0	0

Note: **1**.Total at risk at the beginning of a specific time interval is equal to total at risk for the previous time interval – fatality during that interval. **2**. The fatality rate for positive control group was significantly higher (P value by Fisher Exact test <0.001) than that for each of the 4 vaccination groups.

Table (4-10): Incidence rate of positive culture in selected organs after 7 days of bacterial challenge by study group.

	Total	Positive culture									
		Positive evidence of infection-Air sac		Positive evidence of infection-Liver		Positive evidence of infection-Heart		Positive evidence of infection-Lung		Positive evidence of infection in any site	
Study group	N	N	%	N	%	N	%	N	%	N	%
Control (not inoculated,	5	5	100	5	100	5	100	2	40	5	100

PBL vehicle vaccine)											
Positive control (inoculated but unvaccinated)	5	5	100	5	100	5	100	0	0	5	100
LPS vaccine	5	5	100	4	80	1	20	0	0	5	100

Table (4-21): linear correlation coefficient between selected serum parameters.

	Serum IgM antibodies conc (OD values)	Serum IgG antibodies conc (OD values)	Serum IL6 conc (OD values)	Serum IL1B conc (OD values)
Serum IgG antibodies conc (OD values)	r=0.272 P=0.003			
Serum IL6 conc (OD values)	r=0.56 P<0.001	r=0.331 P<0.001		
Serum IL1B conc (OD values)	r=0.387 P<0.001	r=0.428 P<0.001	r=0.509 P<0.001	
Serum TNF conc (OD values)	r=0.379 P<0.001	r=0.239 P=0.008	r=0.318 P<0.001	r=0.325 P<0.001

Discussion

Extraction and purification of LPS of *E. coli* O78

The standard hot phenol-water extraction method described by Westphal and Luderitz was used to extract LPS from approximately 20 g (wet weight) of *E. coli* O78 and that yield of 200 mcg of crude LPS and to assess the purity of LPS after gel chromatography filtration, from figure of LPS fraction shows one peak of carbohydrate, which that concur to Al-Grawy (1999) who showed only one peak of carbohydrate, but was in contrast to Kshash (2008) and Jann *et al*., (1995) who showed four peaks of carbohydrate then fractions of one peak was selected depending on their high carbohydrate with less level of proteins and nucleic acids as contaminant. This figure of LPS fraction showed one peak of carbohydrate. This result revealed the purity of LPS extraction and purification by hot phenol method, as well as use of EDTA and lysozyme for lyses the cell wall in LPS extraction and added RNase and DNase enzymes led to degrade the nucleic acids which all increased the purity of extracted LPS. Some LPS molecules highly attached to proteins and peptidoglycan which were not passed through gel filtration. LPS is the main component of cell membrane of almost all gram negative bacteria. It is responsible for the pathological consequences of gram negative

bacterial infections . such life threatening diseases as septic shock following infection with gram negative bacteria is mediated mainly by LPS. LPS is potent activator of immune system capable of triggering cytokine release from cells of different origin. It is widely as an inducer of Toll like receptor-4 (TRL-4) signaling pathway and as a mediator of dendritic cell maturation(Kawai and Akira, 2010).

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