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Effect of extracted Lipopolysaccharides of Salmonella typhimurium on chicken immunity

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ABSTRACT

In this study, the prevalence of *Salmonella typhimurium* in Egyptian chicken farms was studied. Seventy-nine chicken farms distributed in Egypt governorates were examined during2017 to 2019. Salmonella was detected in broiler flocks 20 % positivity . However, in layer flocks showed 70 % positivity. Then *Salmonella typhimurium* was confirmed using *Salmonella typhimurium* STM4 495 gene using conventional PCR. Lipopolysaccharides (LPSs) are the major constituent of the bacterial Gram-negative outer membrane including *Salmonella*. Lipopolysaccharides is an immunodominant molecule that is important for the virulence and pathogenesis of *Salmonella typhimurium* which called endotoxin. Extraction and purification of LPS from isolated *S. typhimurium* using isopropanol and sodium hydroxide mixture this overcome the presence of nucleic acids of extracted *Salmonella* in the final product. The purity of LPS isolated from *Salmonella typhimurium* was assessed by HPLC showing highly purified extracted LPS. Effect of extracted LPS of *Salmonella typhimurium* on poultry immunity were studied via a biological experiment using chicks of the broiler breeds to assess the response to LPS stimulation via in vivo experiment using different concentrations of extracted LPS (50, 100, 150 and 200 µg/kg) were injected subcutaneously in 21-day old SPF chicks. After 3 hours of injection, samples were collected to assist the effect of LPS on RNA expression of the immune-related genes, interferon-γ (IFN-γ) and interleukin 1β (IL-1β). There was significant upregulation of IFN-γ and low downregulation of IL-1β of the immune-related genes.

1. INTRODUCTION

Salmonellosis is one of the major enteric diseases in chicken causing mortality and decreased production resulting in high economic losses (Haider et al., 2004). Salmonella is a member of Enterobacteriaceae family. *Salmonella* gallinarum and *Salmonella* pullorum are two sorts of non-motile *Salmonella sp*. that infects poultry causing fowl typhoid and pullorum disease, respectively. Paratyphoid group are motile Salmonella causing salmonellosis in chickens. Salmonellosis is one of the major food borne, zoonotic disease, and remains a potential public health concern worldwide (FAO/WHO, 2009).

Lipopolysaccharides (LPSs) are the major constituent of the bacterial Gram-negative outer membrane (Rietschel et al., 1996). They are known as endotoxins. Some of LPSs functions involve a role in the permeability barrier of the outer membrane (Nikaido, 1979), resistance to phagocytosis and serum (Robertson et al., 1979), work as a receptor for some bacteriophages adsorption (Rapin and Kalckar, 1971), and affect recognition of bacteria (Russa et al., 1982).

LPSs basic structure consists of three parts (Blake and Natividad, 2018): rough core oligosaccharide covalently attached to lipid A and "0" antigen carbohydrate chain (Garidel and Brandenburg, 2009) as shown in fig. 1.

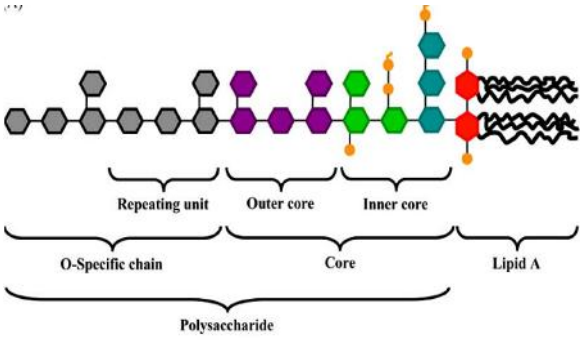

Fig 1. Lipopolysaccharides structure after Garidel and Brandenburg (2009)

Lipid A is highly protected and influence the endotoxic activity, while the ″O″ antigen carbohydrate chain is a polymer of oligosaccharides, which varies between species so used in bacterial serological specificity tests (Erridge et al., 2002).

The Toll-like receptor 4 (TLR4) is important for early host defense against invading microorganisms and for the inflammatory response (Newton and Dixit, 2012). TLR4 recognizes specifically endotoxin LPS leading to the inflammatory response. It can activate neutrophils and macrophages to express several cytokines such as IL-1β,

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TNFα, IL-6, IL-8, and IL-12 (Ramachandran, 2014; Roger et al., 2009).
IL-18 and IL-1β, which are cytokines of the IL-1 family. IL-1β needs inflammatory stimuli for induction, but IL-18 is constitutively expressed. However, the regulation of expression of IL-18 and IL-1β by different inflammatory signals still needs more studies. (Zhu and Kannegant, 2017)
IFN-γ is an inflammatory cytokine, mediating protective immunity to infection and cancer (Masters et al, 2010)
So, this study was aimed to extract LPS of *Salmonella typhimurium* and evaluation of its purity by using HPLC. Then an assessment of its effect on RNA expression of the immune-related genes; interferon-γ (IFN-γ) and interleukin 1β (IL-1β).

2. MATERIAL AND METHODS

2.1. Samples collection:
One hundred Samples were collected from seventy-nine farms (broiler and layers) showing symptoms of Salmonellosis. The birds were obtained between 2017 and 2019. Samples for each flock (cecum, Liver and spleen) were collected, labelled, transported and pooled together as one sample.

2.2. Salmonella isolation, identification, and serotyping:
Salmonella was isolated and identified from the collected internal organs according to ISO 6579-1 (2017) (Media for identification and isolation were purchased from Oxoid).
2.2.1. Media used:
Buffered peptone water (BPW) and Rappaport-Vassiliadis soy peptone broth (RVS) (as pre-enrichment)
Xylose Lysine Deoxycholate Agar (XLD) and Brilliant Green Agar plates (BGA) (Enrichment)
25 gm of each sample was added into 225 ml of buffered peptone water (BPW) as pre-enrichment for 18 h at 37C. Further, 0.1 ml (100 µL) of the pre-enrichment broth were added to 10 ml Rappaport-Vassiliadis soy peptone broth (RVS), incubated at 41.5°C± 0.5°C (18-24 hours). Then, loop full of enriched RVS broth were streaked on Xylose Lysine Deoxycholate Agar (XLD) and Brilliant Green Agar plates (BGA) and incubated at 36.0°C ± 1°C overnight (18-24 hours). Suspected Salmonella colonies were confirmed by using biochemical tests (Oxidase, Indole reaction, Voges Proskauer, Urease hydrolysis test, Methyl red test, Citrate utilization, Lysine decarboxylation test, and H2S production) (Oxoid), Then the confirmed *Salmonella* isolates have been uncovered to serological identity consistent with White Kauffman le Minor Scheme (Kauffman, 1974) for determination of flagellar (H) and somatic (O) antigens.

2.3. Confirmation of Salmonella typhimurium using conventional PCR (Liu et al., 2012):
The isolated *Salmonella typhimurium* strains were confirmed using *Salmonella typhimurium* STM4 495 gene. The DNA was extracted according to (Liu et al., 2012). The sequence of the used primer and amplification conditions were listed in table (1). Separation of PCR products on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at 5V/cm. A one hundred bp DNA ladder was used as a molecular size marker.

*2.4. Extraction of LPS of Salmonella typhimurium (*Fadel and Hassan 2019)
Salmonella colonies suspended in peptone water were centrifuged at 10000 g for 5 min. The settled down pellets were collected and washed twice by Phosphate Buffered Saline (PBS) (pH 7.2). In sterile Eppendorf, 10 mg of the pellets were suspended in 0.5 ml of a mixture consisted of propanol: sodium hydroxide 1 mol/ ml (5:3 v: v) with alkaline pH 11.4. Then the Eppendorf were placed in 100^0 C water bath for 2 hours with gentle mixing by a magnetic stirrer. The mixture was cooled in Freezer at -20^0C and ultra-centrifuged at 10000 g for 15min. The supernatant was collected and the sedimented gel-like layer was extracted by 312.5 µg isopropanol and ultra-centrifuged at 10000 g for 5min. the supernatant was collected and added to the previous one and diluted by the same volume of distilled water. The sample was centrifuged, and the supernatant was collected. The insoluble LPS were extracted by 100 µl methanol (35%) in cold water for 20 min with a magnetic stirrer at 30 rpm. The sample was centrifuged at 2000 rpm for 5min at 4 °C. The supernatant was collected and injected on HPLC.

*2.5. Standard preparation and the chromatographic separation (*Fadel and Hassan (2019)
The purity of LPS isolated from *Salmonella typhimurium* was assessed by HPLC. So, the Stock standard solution was prepared by dissolving 1mg of lyophilized LPS (Sigma-Aldrich L7261, *Salmonella enterica serotype typhimurium*) in deionized water (1ml). Voges Proskauer The best peak resolution was at 3.455 min. as a retention time (RT). The flow rate was 1ml/min and multi-wave detector at a wavelength of 210 nm. The analyte was injected on HPLC with an injection volume 10 µl at room temperature.

2.6. Experimental design and tissue sampling showing the effect of Extracted LPS on poultry: (Xie et al, 2000)
Specific Pathogen Free (SPF) Chicks of the broiler breeds were checked for the response to LPS stimulation. A total of 25 chickens were divided to 5 groups (5/each). At age of 21 days, birds were moved to temperature – humidity-controlled chambers including temperature (25˚C), monitored humidity, wood shaving bedding, water, and corn-soy feed that matched all NRC requirements through the period of the study.
The birds were left for a day to acclimate. Then they were injected subcutaneously with extracted LPS in the amount 50,100,150 and 200 µg/kg, dissolved in (PBS). The negative control group was injected with saline. 3hours post LPS stimulation, chickens were slaughtered. The spleens were immediately collected, put in RNA later which Stabilize and protect RNA with immediate RNase inactivation (Qiagen), and stored at -80˚C until future quantitative measurement of RNA.

2.7. Quantitative measurement of interleukin 1β (IL-1β), interferon-γ (IFN-γ) gene expression:
RNA was extracted from the spleen according to the animal tissue protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). Oligonucleotides Primers and probes were come from Metabion, Germany (table 2).
Analysis of rt-PCR results: Amplification curves and CT values were discovered by the Stratagene MX3005P software. To make an estimation of the variation of gene expression on the RNA of the various samples, the CT of each sample was compared with that of the positive control

group according to the "ΔΔCt" method stated by Yuan *et al*., 2006 using the following ratio: ($2^{-\Delta\Delta ct}$).

3. RESULTS

3.1. Identification of Salmonella isolates from broilers flocks:

The suspected isolates showed the typical colony characteristic of ***Salmonella***. On XLD media, there were red colonies with a black center, and on BGA it appeared as red-pink colonies surrounded by brilliant red zones. The results of microscopical identification of the suspected isolates showed gram-negative, non-sporulated, rod-shaped (bacillus).

Table 1 The sequence of the used primer and amplification conditions.

Target gene	Primers sequences Metabion (Germany)	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles) Secondary denaturation	Annealing	Extension	Final extension	Ref.
S. typhimurium STM4495	GGT GGC AAG GGA ATG AA CGC AGC GTA AAG CAA CT	915 bp	94˚C 5 min	94˚C 30 sec.	50˚C 40 sec.	72˚C 1 min.	72˚C 10 min.	Liu *et al*., 2012

Table 2 Primers sequences, target genes and cycling conditions for TaqMan-PCR.

Target gene	Primers and probes sequences (5'-3')	Reverse transcription	Amplification (40 cycles)			Reference
			Primary Denaturation	Secondary denaturation	Annealing and extension (Optics on)	
28S rRNA	GGCGAAGCCAGAGGAAACT GACGACCGATTTGCACGTC (FAM) AGGACCGCTACGGACCTCCACCA (TAMRA)	50 °C 30 min.	94 °C 15 min.	94 °C 15 sec.	60 °C 1 min.	Suzuki *et al*. (2009)
IFN-γ	GTGAAGAAGGTGAAAGATATCATGGA GCTTTGCGCTGGATTCTCA (FAM) GGCCAAGCTCCCGATGAACGA (TAMRA)					
IL1B	GCTCTACATGTCGTGTGTGATGAG TGTCGATGTCCCGCATGA (FAM) CCACACTGCAGCTGGAGGAAGCC (TAMRA)					Samy *et al*. (2015)

3.2. Isolation of Salmonella species:

The prevalence of ***salmonella*** isolation from all examined 100 samples reached 25%. The incidence of ***salmonella*** infection in the broiler chickens was 20% (18 out of 90), while in the layers was 70% (7 out of 10)

3.3. Biochemical characteristic of *salmonella*:

The biochemical identification of the suspected isolates showed negative reactions for Oxidase (Colorless), Indole reaction (Colorless ring), Vages Proskauer (No bright red color), Urease hydrolysis test (Yellow), and positive reactions for Methyl red test (red color at the surface), Citrate utilization (Development of deep blue color within 24-48 hours), Lysine decarboxylation test (purple color with H2S the middle of the tube), H2S production in triple sugar iron agar (TSI) (Alkaline (red) slant, acid (yellow) bottom with or without blackening due to H2S production).

3.4. Prevalence of *Salmonella typhimurium* recovered from broilers and layers flocks:

The incidence was 8%. Two *Salmonella typhimurium* were isolated from 90 diseased broiler chickens (Table 3).

Table 3 Prevalence of *Salmonella typhimurium* in examined chicken:

Type of flocks	Number of examined chickens	*No. of farms*	*Positive salmonella* isolate		*Positive Salmonella typhimurium isolate*	
			No	**%**	**No**	**%**
Broilers	90	70	18	20	2	8
Layers	10	9	7	70	0	0
Total	100	79	25	25	2	2

3.5. PCR for *Salmonella typhimurium:*

S. typhimurium were confirmed by using conventional PCR Using STM4495 gene of *S. typhimurium.*

3.6. Determination The purity of LPS extracted from *Salmonella typhimurium* using High-performance liquid chromatography (HPLC):

The purity of LPS isolated from ***Salmonella typhimurium*** was checked by HPLC. Then, analysis of the band profile of LPS extracted from *Salmonella typhimurium* and compared to the pure commercial LPS standard. As seen in figure 3, the chromatogram of purified *Salmonella typhimurium* LPS from overlaid with that of standard showing high purity of the product. Chromatogram of purified LPS from *Salmonella typhimurium* also displayed a one sharp band indicating the purity with very low content of impurities.

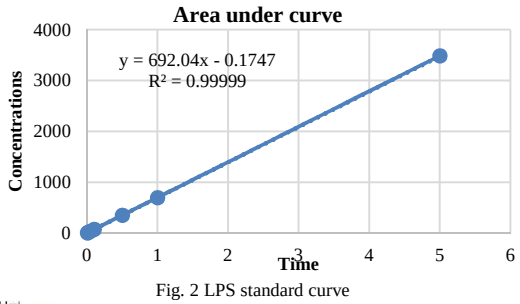

Fig. 2 LPS standard curve

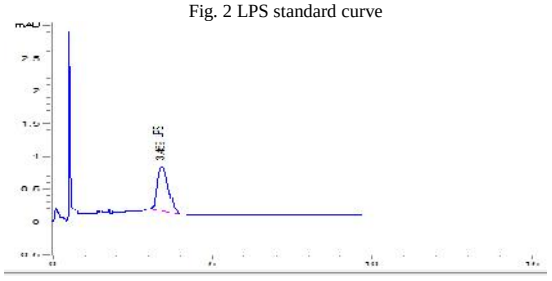

Fig 3 Chromatogram of LPS of Salmonella typhimurium concentration standard (0.005µg/ml).

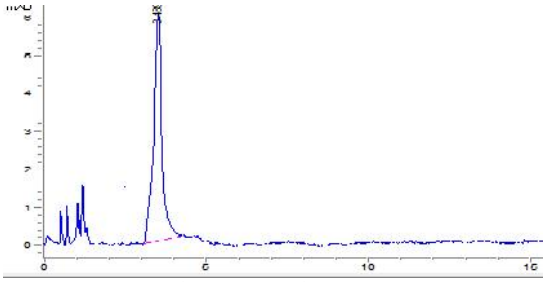

Fig. 4 Chromatogram of extracted LPS from Salmonella typhimurium with concentration (0.06µg/ml).

3.7. Assessment of LPS on Gene expression:

The effect of injected LPS on immunity parameters of SPF chicks were determined by measuring the level of RNA expression of the immune-related genes; interleukin 1β (IL-1β) and interferon-γ (IFN-γ). The result showed elevated RNA expression levels of IFN-γ from the spleen and low-down regulation of IL-1β using different concentrations of LPS (Table 4).

Table 4 The effect of extracted LPS on immunity parameters (IL1β and IFN-γ).

LPS Conc.		IL1β Fold change	*IFN-γ* Fold change
50		0.99	1.07
100		0.95	1.09
150		0.84	1.30
200		0.73	2.12
(0)	Negative group	1	1

4. DISCUSSION

In this study, the prevalence of *Salmonella* among chicken farms in different Egyptian governments was studied. *Salmonella* was detected in broiler flocks; 20 % positivity (18 out of 90). However, in layer flocks showed 70 % positivity (7 out of 10). These results were nearly similar to those reported by Salem et al. (2018), who found that the prevalence of Salmonella in Egyptian broiler flocks was 10.37 % positivity among 3 governorates (Al-Qalyubia, El Ismailia, and El-Gharbia).

In this study Extraction and purification of LPS from isolated *S. typhimurium* were studied. Evaluation of the purity of extracted LPS from different protocols by HPLC analysis. Plenty protocols of LPS extraction and purification were used. The contamination of the end product with excessive content of proteins which could interfere with downstream applications is the main issue with LPS Extraction protocols (Rezania, 2011).

There are many protocols for extraction, separation and purification of LPS were used for example: ether (Ribi et al., 1961), butanol (Morrison and Leive, 1975), Hot-Phenol (Westphal and Jann, 1965), (Goldman and Leive, 1965) proteinase K (Hitchcock and Brown, 1983) and EDTA However impurity, low yield or public health hazard were produced.

The modified method by Mai and Heba, (2019) with propanol and sodium hydroxide mixture yielded LPS recovery on HPLC more than the other two methods (hot phenol and isobutyric acid method). The main proposed reason was explained by Tsang et al. (1974), who said that the cleavage of a phenol-sensitive linkage within the lipid moiety is the reason for the deproteinizing action of hot 45% aqueous phenol on whole cells or isolated and purified endotoxin. This degradation leads to both the LPS and simple protein fragments preserved a part of the lipid moiety. Although not advancing at the same rapid rate as the cleavage of the lipid moiety, such phenol treatment also lead to hydrolysis of the O-specific side chain and ester-bound fatty acids partially.

It was recommended that the presence of the degradation products is one of the major causes of the diversity of endotoxin and LPS preparations.

The evaluation of the LPS extraction by alcohol only precipitate and concentrate LPS fraction as mentioned by Perdomo and Montero (2006). Also, as prescribed by Fadel and Hassan (2019). NaOH was regarded as a suitable chemical to produce LPS with free DNA content. So, the modified method by Fadel and Hassan (2019) for purification of LPS from *Salmonella typhimurium (S. typhimurium)* has high purity and very low contaminating according to new protocol has been conducted.

(Heumann and Roger, 2002) found that LPS is regarded as the large virulence factor of Gram-negative bacteria. The union between LPS and specific Toll-like receptors (TLR) activates the immunomodulatory cells leading to the inflammatory response. (Kogut et al., 2005; Van Dijk et al., 2008) said that this pro-inflammatory response including cytokines such as (IL-1β) and gamma interferon (IFN-γ).

In this study assessment function-ability of LPS through measuring relative levels of RNA expression for the immune-related genes: Interleukin 1 beta (IL-1β), interferon-γ (IFN-γ). The result showed elevated splenic RNA expression levels of IFN-γ and low-down regulation of IL-1β. Leshchinsky and Klasing (2003) and Kaiser et al. (2000) found higher relative levels of immune response gene RNA expression in birds injected with LPS 3 h post-injection than at 24 h post-injection. Xie et al. (2000), Sijben et al. (2003), and Leshchinsky and Klasing (2003) found After LPS administration, there are high concentrations of interleukin (IL)-6 in plasma, and IL-6, IL-1b, IL-18, IL-8, myelo-monocytic growth factor, and interferon-γ in spleen cells. Zeisberger and Roth (1998) and Medvedev et al. (2006) showed that Repeated LPS administration leads to reduce synthesis of cytokines, for example, IL-1b, tumor necrosis factor-a, and IL-6. Wu et al. (2016) found that After LPS administration, there are upregulation of IL-6, IL-1b and chemokine IL-8, the expression of IL-8 and IL-1b was substantially higher than IL-6, and also IL-10 was upregulated similar to IL-1b.

5. CONCULOSIONS

Extraction of LPS by using isopropanol and sodium hydroxide mixture yielded highly purified LPS comparable with standard commercial LPS. Its injection in 21-day old SPF chicks revealed significant upregulation of IFN-γ and low downregulation of IL-1β of the immune-related genes.

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