Iraqi Journal of Veterinary Sciences, Vol. 35, Supplement III, 2021 (149-155)
Proceedings of the 13th (2nd International) Scientific Conference, College of Veterinary Medicine, University of Baghdad

Iraqi Journal of Veterinary Sciences

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Immunotherapeutic effect of chitosan and listeriolysin O on *Listeria monocytogenes* infection in mice

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Article information

Article history:
Received November 27, 2021
Accepted December 17, 2021
Available online December 11, 2021

Keywords:
L. monocytogenes
listeriolysin O
Chitosan
IL-6
IgG

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Abstract

This study aimed to evaluate the effect of dietary chitosan and listeriolysin O (LLO) on the immune response against *Listeria monocytogenes* infection in mice. *L. monocytogenes* isolate was obtained from the unit of Zoonotic Disease, College of Veterinary Medicine, University of Baghdad. It was reactivated and cultured on PALCAM agar and exposed to a confirmatory diagnostic test. listeriolysin O (LLO) was extracted and purified. Sixty mice were used and divided into four groups each one involving 15 mice; the first group was fed on diet supplemented with a chitosan for 4 weeks. At the end of the 2nd week, normal saline was injected S/C; the second group was fed on a normal diet without any addition. At the end of 2nd week, 0.3 ml of LLO was injected S/C, then the booster dose of LLO was given after 14 days of 1st injection; the third group was fed on a diet supplemented with chitosan as in G1, and treated as in G2 and the fourth group fed on a normal diet without any addition and treated as in G1. A skin test was performed on 5 mice of each group while a Challenge test by injection of 0.2ml of $1x10^9$ CFU/ml of viable *L. monocytogenes* intraperitoneally was performed on other mice. The concentration of IgG titer and IL6 were measured. The results revealed that the third group recorded significantly higher values in the skin thickness, IgG, and IL6 concentrations when compared with other groups at different times which indicate that LLO and chitosan may support each other to provide the most beneficial effect by eliciting of good cellular and humeral immunity against listeriosis.

DOI: 10.33899/ijvs.2021.132332.2082,

Introduction

Listeria monocytogenes is a Gram-positive, non-spore-forming, motile, facultative anaerobic short rod about 0.2 × 0.5 to 2 µm that is widely distributed in nature and is frequently isolated from a variety of sources, including soil, mud, decaying vegetation, contaminated silage, fecal materials, and food, particularly raw, unpasteurized milk and its products. These bacteria are an intracellular pathogen that causes listeriosis in humans and other mammals, a serious public health concern. Listeriosis is, without a doubt, one of the most dangerous foodborne zoonoses, life-threatening disease with a fatality rate of 20-30% (1-3).

The hemolysin listeriolysin O (LLO), the phosphatidylinositol-specific phospholipase C, the actin assembly-inducing protein (ActA protein), positive regulatory factor A (prfA), and extracellular protein (P60) are all virulence factors produced by *L. monocytogenes* (4). LLO is an immunogenic protein that will eventually be utilized by the immunocompetent host to activate immune cells and eliminate the pathogen permanently. LLO is an immunomodulatory molecule in addition to supplying key antigens.

The molecular mechanism for LLO's adjuvant characteristics is unknown, but it underscores LLO's importance in the regulation of innate and adaptive immune responses (5). Chitin is a polysaccharide found in fungal cell

walls, crustacean and insect exoskeletons, and is the second most prevalent biopolymer after cellulose in nature. Deacetylation is the process of converting chitin tochitosan by removing the acetyl group. Because of its biological features of biocompatibility, biodegradability, and atoxicity, chitosan is widely employed as a biomaterial.

Because it contains antibacterial and antifungal properties, it could be employed as a therapeutic agent in agriculture, medicine, the environment, as well as the food, cosmetic, and textile sectors (6). Chitosan has a strong immunostimulant effect, dietary administration of chitosan in mice showed enhancement of both humoral and cell-mediated immunity (7).

The present study aimed to evaluate the effect of dietary chitosan and listeriolysin O on *L. monocytogenes* infection in mice.

Materials and methods

Source of *Listeria monocytogenes* isolate

The bacterial isolate was obtained from the unit of Zoonotic Disease /College of the Veterinary Medicine, University of Baghdad. Then, it was reactivated by transferring a loopful isolate from slant agar and culturing on brain heart infusion broth and incubated at 37°C for 18 hrs, followed by culturing on selective listeria agar (PALCAM) and exposed to confirmatory diagnosis by microscopic examination and biochemical tests (oxidase, catalase, hemolysis on blood agar, motility test) and VITEK 2 system.

Extraction and purification of listeriolysin O

Listeria monocytogenes was cultured in one liter of tryptic soya broth and incubated for 18 hours at 37°C, then the cultured broth was centrifuged by cooled centrifuge in (6000) RPM for 45 min at 4°C, after that the supernatant was collected which represent the crude hemolysin in sterile flasks and filtered by Millipore filter membrane (0.22 µm) followed by estimation of hemolytic activity and the protein concentration of the crude hemolysin (8), then subjected to ion-exchange chromatography using DEAE-cellulose column as described by (9).

In a further purification step gel filtration chromatography on Sephadex G-1500 (Pharmacia) has been done (10), and fractions were tested for hemolytic activity. Active fractions were pooled and stored until use. The LLO purity was determined by SDS-PAGE.

Estimation of hemolytic activity assay

Estimation of the hemolytic activity was performed according to Wang *et al.* (11), A 875 µl of hemolysis buffer, 100 µl of bacterial culture supernatant (crude hemolysin), and 25 µl of sheep erythrocytes were mixed and incubated at 37°C for 30 min. the mixture was centrifuged at 5500 rpm for 1min, then the supernatant was used to examine the hemolytic activity by determining the optical density of each sample at 543 nm. The samples were treated with 1% Triton X-100 to achieve 100% hemolysis, and the percentage of hemolysis was measured by comparing each sample to the control culture.

Experimental study

Sixty white Swiss Balb mice of both sex, aged 8-9 weeks and weighed 30-35 gm were obtained from national center for drug control and research / Baghdad. They were housed and maintained in the animal house of the College of Veterinary Medicine with controlled conditions of temperature at 22±5°C and light. They were housed in plastic cages containing hard-wood chips as bedding which was changed constantly to ensure a clean environment. The animals were adapted for one week before starting the experiment they were fed on special formula of standard pellets and water. The animals were divided into four groups; the first group involved 15 mice fed on a diet supplemented with chitosan (1.5 gm/kg diet) for 4 weeks.

At the end of the 2nd week, normal saline was injected S/C, then after 14 days of the 1st injection, normal saline was injected, the second group involved 15 mice fed on a normal diet without any addition. At the end of the 2nd week, 0.3 ml of LLO was injected S/C, then the booster dose of LLO was given after 14 days of 1st injection, the third group involved 15 mice fed on a diet supplemented with chitosan 1.5 gm/kg diet for 4 weeks**.** At the end of the 2nd week, 0.3 ml of LLO was injected S/C, then the booster dose of LLO was given after 14 days of 1st injection, and the fourth group involved 15 mice fed on a normal diet without any addition. At the end of the 2nd week, normal saline was injected S/C, then after 14 days of the 1st injection, normal saline was injected.

After 7days of the booster dose, skin test was performed on five mice from each group by injection of 0.1 ml of LLO intradermally in the footpad of mice, while the other remaining mice were exposed to the challenge dose of *L. monocytogenes* culture by injection of 0.2ml of $1*10^{9}$ CFU/ml intraperitoneally.

Results

Identification of *L. monocytogenes*

The bacterial growth on PALCAM appeared as gray-green colonies with black sunken center and black halo against a cherry red background, about 2 mm in diameter with blackening of the medium (Figure 1), Gram-positive short rods, cocci or filamentous, and arranged in single, pair or (V and Y) shapes know as palisade shapes under light microscope (Figure 2); narrow, clear and light zones of hemolysis (ß-haemolysis) on blood agar, oxidase negative, catalase- positive and motile (tumbling) at 25°C. Vitek®2 system was used to confirm the diagnosis of *Listeria monocytogenes* isolate.

Figure 1: Characteristics feature of *Listeria monocytogenes* colony on PALCAM media.

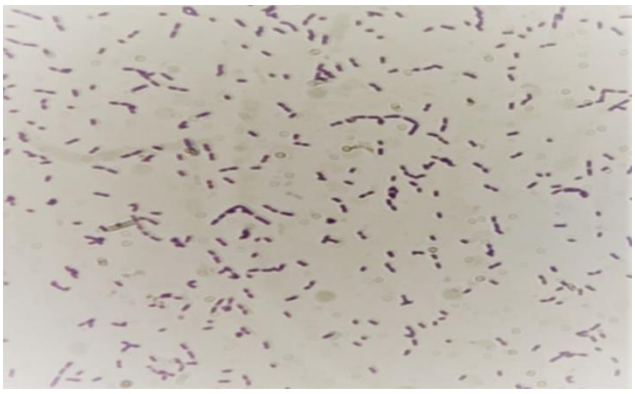

Figure 2: Gram stain of *Listeria monocytogenes* showed gram- positive, short rods, single or in chain, with characteristic V and Y shapes

Purification of LLO

The results of the ion-exchange chromatography showed the appearance of one protein peak and one peak of the hemolytic activity of listeriolysin O on the separated fractions 65-71 (Figure 3), these fractions were collected and subjected to Sephadex G-1500 gel filtration column for further purification that showed the appearance of one protein peak and one peak of the hemolytic activity 91% of listeriolysin O on the separated fractions 16-27 (Figure 4). The fractions that had hemolytic activity were collected and the protein concentration was measured 16 mg/ml and stored until used in the experiment. The molecular weight of LLO was determined by SDS-PAGE depending on the bands of a standard ladder Tris-Glycine (Figure 5), resulting in the appearance of two protein bands were approximately 58 KDa.

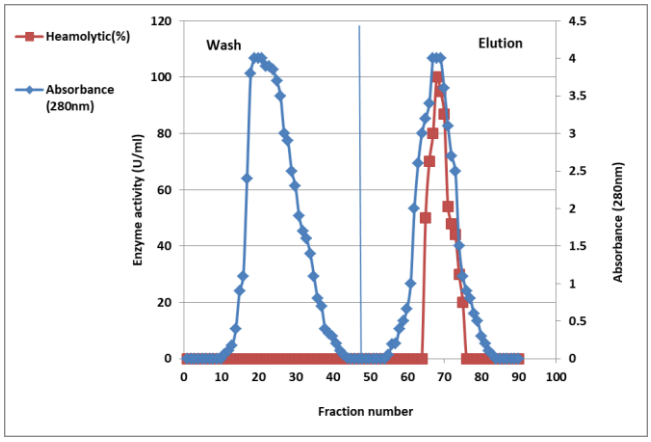

Figure 3: Ion-exchange chromatography for the purification of listeriolysin O from *L. monocytogenes* using a column ion-exchange DEAE-cellulose with dimensions 2*25cm equilibrated with 0.05 M sodium phosphate buffer solution at pH 7, and recovered with a phosphate buffer solution with saline gradient 0.1-1 Molar NaCl at a flow rate of 30 ml/hr and at a rate of 5 ml/part.

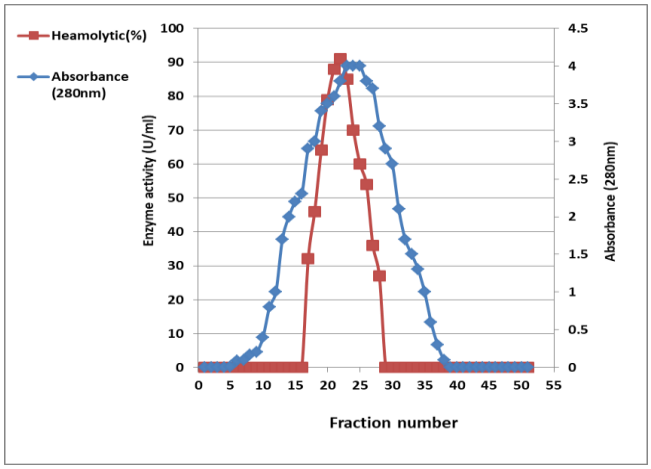

Figure 4: Gel filtration chromatography to purify listeriolysin O using a Sephadex-1500 gel column with dimensions 2*35 cm equilibration and recovery with 0.05 M sodium phosphate buffer solution at pH7, flow rate 30 ml /hour and 5 ml/part.

Delayed- Type Hypersensitivity reaction (DTH)

The present study explained that in the second and third group LLO and CS+LLO, the mean value of skin thickness 2.30±0.17, 3.00±0.40 respectively was higher significantly than those in the first CS and control group 1.20±0.10, 1.00±0.10 respectively and there was no significant difference between the 1st and 4th group at 24hrs. But at 48hrs., the value of skin thickness in the third group CS+LLO was more elevated when compared with other groups (Table 1).

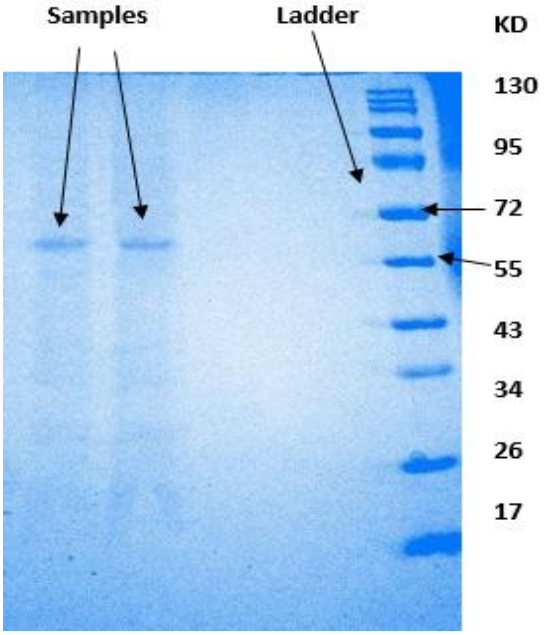

Figure 5: 10% SDS- polyacrylamide gel electrophoresis for listeriolysin o protein using tris-glycine ladder.

Table 1: Mean difference in skin thickness (mm) in all groups of mice

Groups	Mean ± SE of Skin test		LSD
	24 Hours	48 Hours	
CS	1.2±0.1 Bb	2.5±0.2 Ba	0.373 *
LLO	2.3±0.1 Aa	3.0±0.3 Ba	0.616 NS
CS+LLO	3.0±0.4 Aa	3.9±0.6 Aa	1.29 *
Control	1.0±0.1 Ba	0.2±0.1 Cb	0.286 *
LSD	0.725 *	0.610 *	---

Means with different small letters in the same column and big letters in the same row are significantly different at P≤0.05*.

Determination of the level of IL6 concentration

The result of IL6 concentration expressed an elevated mean value in the third group CS+LLO with a significant difference at the 14th and 21st day 17.80±0.24, 24.80±0.37 respectively, higher than those of the second group LLO which was recorded 6.60±0.21, 14.15±0.13, 9.15±0.40 respectively, as compared to control group 3.20±0.22, while the CS group 3.90±0.33, 4.30±0.21, 3.82±0.32 respectively showed non-significant difference as compared to the control group (Table 2).

Table 2: The difference in IL6 concentration mean in all groups of mice

Groups	Mean ± SE of Interleukin 6			LSD
	14th Day	21th Day	28th Day	
CS	3.9±0.3 A c	4.3±0.2 A c	3.8±0.3 A c	0.533 NS
LLO	6.6±0.2 C b	14.1±0.1 A b	9.1±0.4 B b	1.262 *
CS+LLO	17.8±0.2 B a	24.8±0.3 A a	13.5±0.2 C a	1.407 *
Control	3.2±0.2 A c	3.2±0.2 A d	3.2±0.2 A c	0.723 NS
LSD	0.795 *	0.766 *	0.928 *	---

Means with different small letters in the same column and big letters in the same row are significantly different at P≤0.05*.

Determination of IgG concentration

By using radial immunodiffusion plates, the immunoglobulin G concentration was detected, in which the antibody precipitating ring of each well was measured by an appropriate ruler. The finding of the present study showed a significant difference between groups.

The results of the present study showed an elevated level of IgG concentration in the third group LLO+CS at the 14th, 21st and 28th days of the experiment 10.00±1.08, 12.00±0.40, 14.00±0.81 respectively as compared to the control group 5.00±0.41, 5.00±0.42, 5.00±0.40 respectively and the other groups, while both the first CS and the second LLO groups showed a significant difference in day 21st and 28th which recorded 7.00±0.40, 9.00±0.73 and 8.00±0.73, 11.00±1.08 respectively with a non-significant difference at the day 14th 4.50±0.64, 6.50±0.64 when compared to the control group (Table 3).

Table 3: The difference in IgG concentration mean in all groups of mice

Groups	Mean ± SE of IgG			LSD
	14th Day	21th Day	28th Day	
CS	4.5±0.6 B c	7.0±0.4 B b	9.0±0.7 B a	1.95 *
LLO	6.5±0.6 B b	8.0±0.7 B b	11.0±1.0 B a	2.69 *
CS+LLO	10.0±1.0 A b	12.0±0.4 Aa b	14.0±0.8 A a	2.61 *
Control	5.0±0.4 B a	5.0±0.4 C a	5.0±0.4 C a	1.30 NS
LSD	2.267 *	1.572 *	2.450 *	---

Means with different small letters in the same column and big letters in the same row are significantly different at P≤0.05*.

Discussion

We did not found sufficient references that directly related or close to our work, despite the availability of references related to chitosan effect on other animal species or microorganism and forms such as nanoparticles, mostly chitosan and LLO used as adjuvants; so the present study may represent an original study.

Mammalian hosts have several redundant mechanisms to respond quickly in the event of infection, including innate immune response which is generated within minutes to hours after infection, and the adaptive immune response that is essential to reach sterilizing protective immunity (12). *L. monocytogenes* infection can activate both the innate (neutrophils and macrophages) and adaptive (CD4+ and CD8+ T cells) immune responses (13). Furthermore, infected cells and associated immune cells produce a variety of cytokines and chemokines, including IL-1, IL-6, IL-12, CC chemokine ligand 2 (CCL2), interferon (IFN)- β and tumor necrosis factor (TNF)- α that activate APCs, eliciting an innate immune response and stimulating a T-helper 1 (Th1) cell-mediated immune response (14).

Listeriolysin O is a pore-forming toxin whose primary purpose is to allow cytosolic bacterial multiplication by breaching phagosomal membranes, which is important for the pathogen to elude host immunological identification (15).

As delayed-type hypersensitivity is the primary pattern of cell-mediated immunity, the results of this study showed that the LLO and chitosan utilized in this investigation triggered both cellular and humoral immune responses. Delayed type hypersensitivity is an important defense mechanism counter to intracellular infections such as mycobacteria, fungi, and certain parasites that occurs at least 48 hours after antigen exposure (16).

There are very few studies that used LLO as an antigen to measure DTH. However, Barbuddhea *et al.* (9) injected purified LLO intradermally in calves and the skin induration against LLO was found to be maximum at 24 hrs. and it decreased subsequently, that result disagreed with the present study in which the skin thickness reached the maximum at the 48 hrs of injection. Wardani *et al.* (17) found that chitosan nanoparticles induce an increase in DTH response in rats, shows a stimulatory effect of the chitosan nanoparticles which has happened on the lymphocytes and accessory cell types essential for the expression of this reaction and this with an agreement with the present study that showed gradual increase in induration of skin thickness. Since both LLO and chitosan have an immunostimulatory effect and can induce cell-mediated immunity and the activated T cells produce cytokines and chemokines, which attract macrophages and cytotoxic CD8+ T cells may explain the elevated mean value ($P \leq 0.05$) skin thickness in the CS+LLO group as compared to the CS and LLO group as mentioned by Carrero *et al.* and Li *et al.* (18,19).

IL-6 is a significant modulator of acute-phase proteins that regulates neutrophil and monocyte responses in the aftermath of an infection, IL-6 is a lymphocyte survival factor that also increases B cell Ab production. IL-6 has been known as a critical cytokine for the development of CD4+ T cells into Th17 cells, a main proinflammatory T cell population (20). The role of LLO in IL-6 expression in Caco-2 cells during *L. monocytogenes* infection in vitro was investigated by Tsuchiya *et al.* (21). They discovered that wild-type *L. monocytogenes* promoted persistent IL-6 production in Caco-2 cells, but non-LLO-producing strains only induced transitory IL-6 expression.

The result of the present study showed that IL6 production in response to both LLO and chitosan was reached a maximum at the day 21 post-immunization may due to the activation of macrophages that release a diversity of proinflammatory mediators and cytokines comprising interleukin-6, activate other cells to initiate inflammation as mentioned by Yang *et al.* (22). Chitosan has been shown to stimulate macrophages, enhance cell proliferation, and activation, and hence modulate innate and adaptive immune responses.

Antibodies to particular immune responses to antigens mediate humoral immunity. Neutralization, antibody-dependent cellular cytotoxicity, complement activation and opsonization are the main biological actions of antibodies (23).

This study examined the effects of LLO and CS on the immune function, the results showed an elevated level of IgG concentration in the third group LLO+CS at the 14^{th}, 21^{st} and 28^{th} days of the experiment indicate that LLO and chitosan may support each other to provide the most beneficial effect by eliciting of good humoral immunity, this finding was in agreement with a study performed by Rekha *et al.* (24) in experimentally infected goat with pathogenic *Listeria monocytogenes* in which antibodies to LLO appeared by days 10-14 post-infection, peaked between days 11 and 25 days post-infection and Abd-ElTawab *et al.* (25) showed that the water supplementation of chitosan in mice induced a significant increase in the level of IgG.

Conclusion

The results revealed that the third group recorded significantly higher values in the skin thickness, IgG, and IL6 concentrations when compared with other groups at different times which indicate that LLO and chitosan may support each other to provide the most beneficial effect by eliciting of good cellular and humeral immunity against listeriosis.

Acknowledgment

The authors would like to acknowledge all the staff of the Microbiology department for their support, as well as the deanery of the College of the Veterinary Medicine,

University of Baghdad for organizing and holding this scientific conference.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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تأثير العلاج المناعي للكيتوسان ولستريولايسين على الإصابة بجرثومة الليستيريا المستوحدة في الفئران

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الخلاصة

هدفت الدراسة إلى تقييم فعالية الكيتوسان الغذائي والليستريولايسين على الاستجابة المناعية ضد الإصابة بجرثومة الليستيريا المستوحدة في الفئران. تم الحصول على العزلة البكتيرية من وحدة الأمراض المشتركة في كلية الطب البيطري، جامعة بغداد وتم إعادة تنشيطها وزراعتها على وسط البالكام وتم إجراء الاختبارات التشخيصية لها. تم استخلاص وتنقية الليستريولايسين. استخدم بالتجربة ٦٠ فأرا وقسمت الفئران إلى ٤ مجاميع تضم كل مجموعة ١٥ فأرا. غذيت حيوانات المجموعة الأولى بعلف مكمل بالكيتوسان لمدة ٤ اسابيع وفي نهاية الأسبوع الثاني تم حقن محلول دارئ الفوسفات تحت الجلد. المجموعة الثانية كانت تتغذى على علف خالي من أي إضافات وفي نهاية الأسبوع الثاني تم حقنها ب ٠,٣

مل من الليستريولايسين تحت الجلد ثم أعطيت الجرعة المنشطة بعد ١٤ يوما من الجرعة الأولى لليستريولايسين. المجموعة الثالثة غذيت بعلف مكمل بالكيتوسان كما المجموعة الأولى ثم عوملت بنفس طريقة المجموعة الثانية. أما المجموعة الرابعة فقد غذيت على علف خالي من الإضافات ثم عوملت بنفس طريقة المجموعة الأولى. أجري الفحص الجلدي ل ٥ فئران من كل مجموعة بينما اجري فحص التحدي على الفئران الأخرى. تم قياس تركيز معيار الكلولبيولين المناعي ج والانترلوكين السادس على فترات مختلفة. أظهرت النتائج أن المجموعة الثالثة سجلت قيما معنوية أعلى بشكل ملحوظ في سمك الجلد وتراكيز كل من الكلولبيولين المناعي ج والانترلوكين السادس عند مقارنتها بالمجاميع الأخرى في أوقات مختلفة مما يشير إلى أن الكيتوسان والليستريولايسين قد يدعمان بعضهما البعض لتوفير التأثير الأكثر فعالية في إظهار الاستجابة المناعية الخلطية والخلوية ضد الإصابة باليستيريا.