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The effect of *Citrobacter fruendii* LPS on the immune parameters of rabbits immunized with *Serratia marcescens* antigens

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Abstract: *Serratia marcescens* is a significant opportunistic pathogen that affects a variety of hosts, including invertebrates, plants, and mammals. It is linked to a wide range of clinical illnesses, including pneumonia, keratitis, meningitis, urinary tract infections, and wound infections. The present research aimed to estimate the immune response of rabbits immunized with whole cell sonicated antigen of *Serratia marcescens*, killed whole cell antigen of *S. marcescens* and lipopolysaccharide extracted from *Citrobacter freundii* against *Serratia marcescens* infection; a challenge dose was $8x10^6$ CFU/ml. Thirty five (35) white male rabbits aged between 6-8 weeks, were divided randomly into five groups (7 rabbits each) and ELISA test was performed. The first group received a subcutaneous (S/C) immunization with WCSAg-S and LPS-C (1000 g/mL) vaccine. The second group received KWCA-S (9×10^8 CFU/mL) and LPS-C (1000 g/mL) vaccinations. The sole vaccine given to the third group was WCSAg-S (1000 g/mL). LPS-C (1000 g/ml) was used to immunize the fourth group. 1 mL of PBS was given subcutaneously into the fifth group (the negative control) (pH 7.2). The rabbits received booster doses of the same antigens after 14 days. Following 21, 35, 49, and 53 days of vaccination, the inoculated animals demonstrated significant changes ($P<0.05$) in IL-2 and IL-10 compared to the negative control group. In conclusion, the 3rd group immunized with WCSA-S only was able to promote stronger humoral and cellular immune responses against *Serratia marcescens* infection in comparison with the other groups.

Keywords: *Serratia marcescens*, *Citrobacter freundii*, antigens, ELISA, LPS

Introduction

Global nosocomial pathogen *Serratia marcescens* is an opportunistic pathogen of numerous plants and animals, including humans. Under certain circumstances, such as disruption of the normal gut microbiota or the presence of Varroa mites that puncture the membrane to allow entry of the bacterial cells, it may be highly virulent. (1).This bacterium may be recovered from a variety of clinical specimens, such as blood, tracheal aspirates, and urine, when it is linked with infection. The organism is only pathogenic when present in the circulation (2). *Citrobacter* spp. are zoonotic pathogens and opportunistic nosocomial bacteria that can infect sheep with encephalitis, septicemia, and respiratory tract infections (3,4). The natural adjuvant lipopolysaccharide (LPS), which is derived from the cell membrane of Gram-negative bacteria, works in part by creating an inflammatory environment during lymphocyte activation. When adjuvants are added, the tolerogenic signal is quickly transformed into an effective immune response during both primary and secondary exposures. (5). Both Lipid A, the lethal component of LPS, and the polysaccharide side chains, the harmless but immunogenic component of LPS, function as indicators of pathogenicity in Gram-negative bacteria. Antigen protection, phagocyte resistance, and antigenic diversity are just a few of the traits that O-antigens possess. Lipid A stimulates the immune system, causing a particular organism's biological reactions (6). Gram-negative bacterial infection stimulates the humoral immune response and the synthesis of immunoglobulins from B cells, including IgM and IgG, as well as the systemic and local immunological responses. CD4+ T cells play a key part in these immune responses. These immunoglobulins assist this bacterium's opsonization process, which aids in phagocytosis' ability to destroy it (7). One of the most

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crucial regulators of vital activity is interleukin-2 (IL-2), which controls the growth and differentiation of naive T lymphocytes into effector T cells, the rise in NK cells' cytolytic activity, the proliferation of B lymphocytes, the secretion of immunoglobulin, and other processes (8,9). Interleukin-10 (IL-10) is an anti-inflammatory cytokine that inhibits Th1 responses and the production and/or activity of a number of inflammatory cytokines in monocytes and T cells, including IFN-, TNF, IL-1, and IL-6. Additionally, it has been shown to promote immunological processes such B cell immunoglobulin synthesis, NK cell and CD8+ T cell cytotoxicity, and thymocyte proliferation; as a result, IL-10 has been described as exhibiting a dual nature in the immune response: both repressive and promoting (10). Due to the limited studies about *S. marcescens* that presented in the social environment of human and animals, we conducted this research to study the effect of *Serratia marcescens* antigens associated by *Citrobacter fruendii* LPS on some immune parameters in rabbits

Materials and methods

- **Bacterial isolation**

Serratia marcescens was isolated from fecal sheep samples in Baghdad Provence; each sample was plated onto MacConkey's and Nutrient agar and incubated for 24 to 48 hours at 37 °C. Utilizing the conventional morphological and biochemical assays, the isolates were identified. Citrobacter freundii was acquired from the microbiology division of the University of Baghdad's College of Veterinary Medicine.

- **Antigen preparation**

The whole cell sonicated antigen of *S. marcescens* (WCSA-S) was prepared according to Motive, 1992 (11). Killed whole cell antigen of *S.marcescens* (KWCA-S) was prepared according to Motive (11). While, the LPS antigen was prepared according to Johnson and Perry (12).

- **Determination of LPS Carbohydrate and protein:**

The carbohydrate concentration was evaluated using the phenol-H_2SO_4 technique originally reported by Dubois *et al*., (1956) (13); The protein content was computed from the standard curve in accordance with Bradford, (1976) (14).

- **Rabbits immunization**

Thirty five male Albino rabbits of 2-3 kg were divided randomly to five groups (7 rabbits for each), as follows: The 1st group was immunized with WCSA-S +LPS-C (1000 µg/mL, S/C). The 2nd group was immunized with KWCA-S ($9x10^8$) + LPS-C (1000 µg/mL S/C). The 3rd group was immunized with WCSA-S (1000 µg/mL, S/C). The 4^{th} group was given LPS-C (1000 µg/mL, S/C).

Antigen booster dose of 1 mL was given to the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} groups on day 14 post immunization.

ELISA test:-

Blood samples were taken on days 21, 35, 49, and 53 following vaccination, and the sera were then divided to estimate the concentrations of IL-2 and IL-10 using ELISA kits: IL-2 and IL-10 (Elabscience, China).

Statistical Analysis:-

The impact of various factors on the research parameters was determined using the SAS (2012) software, which does statistical analysis. To compare between means, the least significant difference test (LSD, Analysis of Variation-ANOVA) was employed (15).

Results:

All vaccinated groups' findings for IL-2 concentration revealed a substantial rise in IL-2 levels with discernible variances ($P<0.05$) at 21, 35, 49, 53 days post immunization in comparison with the control group as shown in Figure (1). The concentration of IL-2 in the first group, reached 4.68± 0.82 pg on day 21 post immunizations, then elevated to reach 5.41± 1.30; 4.11± 0.28 and 8.80±0.39 pg at 35, 49, 53 days respectively post immunization.

In the second group, the results showed significant differences ($P<0.05$) on the day 53 compared with the days 21, 35 and 49. The concentration of IL-2 was 5.19± 0.91, 4.99± 0.59, 3.97± 1.38 and 6.31± 2.59 pg after 21, 35, 49 and 52 days, respectively, post immunization of rabbits, These results showed significant differences ($P<0.05$) at day 53 in comparison with day 21, 35 and 49.

The third group of rabbits showed the highest and statistically significant IL-2 concentration (6.78± 1.43 pg, $P<0.05$) on day 35 compared with the findings of day 49.

The level of IL-2 of the fourth group following 21 days of immunization represented the highest and significant value (7.71± 2.04 pg, $P<0.05$) compared with other treated groups (5.56± 0.54, 5.55± 1.36 and

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4.73± 0.56 pg. after 35, 49 and 53 days, respectively, of immunization. The control group (PBS), showed the lowest concentrations of IL-2 (4.26± 0.74; 5.56± 0.79; 4.94± .412 and 3.49± 1.02) after 21, 35, 49 and 53 days of immunization and the levels remained low till the end of the experiment. There were significant differences (P<0.05) between the 1st, 2nd, 3th and 4th groups compared with the control.

Regarding the concentration of IL-10 in the first group, it reached a significant level (P<0.05) of 77.58± 2.16 pg. on day 21, then decreased to reach 35.39± 9.26, 50.72± 10.81 and 76.14± 5.45 pg. on the day 35, 49 and 53, respectively, post immunization of rabbits. The second group revealed that the concentrations of IL-10 were 53.94± 5.17, 42.51± 13.70, 51.90± 2.77 and 81.12± 6.07 pg. after 21, 35, 49 and 52 days respectively post immunization. These results were statistically significant (P<0.05) on day 53 in comparison with the findings of the other days. The third group of rabbits showed that the IL-10 concentrations were 70.94± 5.24, 32.65± 6.16, 54.46± 4.08 and 66.73± 4.18 on day 21, 35, 49 and 53, respectively, after immunization with significant differences (P<0.05) on day 21 in comparison with the other days. The 4th group showed that the IL-10 concentrations were 61.45± 6.16, 37.98± 4.33 and 77.24± 5.18 pg. on day 21, 35 and 49 respectively while the concentration reached the highest level (116.44± 8.90 pg.) on day 53. Finally the control group showed low IL-10 concentrations of 57.05± 2.06, 36.74± 6.28, 64.60± 3.78 and 67.92± 4.38 after 21, 35, 49 and 53 days respectively of immunization.

There were significant differences (P<0.05) between the 1st, 2nd, 3rd and 4th groups relative to the control group.

Time / groups	Mean ± SE			
	21 day	**35 day**	**49 day**	**52 day**
1st group WCSA-*S*+ LPS-C (1000µg/m	**4.68± 0.82 AB b**	**5.41± 1.30 A b**	**4.11± 0.28 A b**	**8.80± 0.39 A a**
2nd group KWCA-*S*+ LPS-C (500µg/ml)	**5.19± 0.91 AB ab**	**4.99± 0.59 A ab**	**3.97± 1.38 A b**	**6.31± 2.59 A a**
3th group WCSA-*S*	**5.40± 0.82 AB ab**	**6.78± 1.43 A a**	**4.43± 0.74 A b**	**4.69± 0.96 AB ab**
4th group LPS-C	**7.71± 2.04 A a**	**5.56± 0.54 A b**	**5.55± 1.36 A b**	**4.73± 0.56 AB b**
5th group PBS	**3.26± 0.74 B a**	**2.56± 0.79 B a**	**2.94± 0.41 B a**	**3.49± 1.02 B a**

Figure (1): IL-2 concentration tested by ELISA test in the animals immunized with different antigens.

Time / groups	Mean ± SE			
	21 day	**35 day**	**49 day**	**52 day**
1st group WCSA-*S*+ LPS-C	77.58± 2.16 A a	75.39± 9.26 A b	50.72± 10.81 B ab	76.14± 5.45 B a

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(1000µg/ml				
2nd group KWCA-*S*+ LPS C(500µg/ml	53.94± 5.17 A ab	52.51± 13.70 A b	51.90± 2.77 B ab	81.12± 6.07 B a
3th group KWCSA-*S*	70.94± 5.24 A a	72.65± 6.16 A b	54.46± 4.08 B ab	66.73± 4.18 B ab
4th group LPS-C	61.45± 6.16 A b	77.98± 4.33 A b	77.24± 5.18 A b	116.44± 8.90 A a
5th group PBS	37.05± 2.06 B ab	36.74± 6.28 B b	34.60± 3.78 C ab	37.92± 4.38 C a

Figure (2): IL-10 concentration tested by ELISA test in the animals immunized with different antigens

Discussion:

Serratia marcescens is an important opportunistic pathogen associated with a wide spectrum of clinical diseases, this research was conducted to study the immune parameters associated with *Citrobacter fruendii* LPS; because the latter is a potent immunomodulator and acts on lymphoreticular cells, e.g., B cells, macrophages (Mø), and T cells. *Citrobacter freundii* has O (somatic) cell wall antigen, O-specific polysaccharide antigens in LPS, which contain similar epitopes with other Gram- negative bacteria involving N-acyl or N-acetyl derivatives of 4-amino-4,6-dideoxy-K-D-mannopyranosyl residues (16, 17). Interlukin-2 decreased in all first three immunized groups and then elevated at day 52. *Serratia marcescens* produces a characteristic red pigment, prodigiosin as a virulent factor (18). This result was confirmed by (19) who found that Prodigiosin has been shown to have immunosuppressive effects and to inhibit the growth of T cells while having no impact on B cells. It works by preventing the interleukin-2 receptor (IL-2R) chain, a key factor in T-cell activation, from being expressed. Authors of a different study created a prodigiosin analogue molecule that inhibited both T-cell and B-cell activation (20). The results of AL-Taee and Al-Samarraae (21), who examined the impact of crude, sonicated, and P. aeruginosa lipopolysaccharide antigens on immunological markers (IL-2), in female albino rats, show that IL-2 of sonicated was 66-74%, LPS 90-60%, and killed 27-60%. These findings are consistent with this study. Injecting LPS combined with peptide lowered IL-2 levels and secondary clonal development, showing that LPS produced components that inhibited the T cells. Additionally, this result was in keeping with McAleer and Vella's (5) study that LPS can also adversely affect Th function. This also supports the findings of Han *et.al*, (22) who discovered that prodigiosin functioned by suppressing IL-2 dependent signaling, rather than by preventing IL-2R induction or activation of AP-1 and NF-B, prodigiosin was also synergistically active when administered with cyclosporine A, each working through different pathways to suppress T-cell activation.

IL-10 is an anti-inflammatory cytokine that can prevent macrophages and polymorphonuclear cells from secreting pro-inflammatory cytokines. The development of T regulatory 1 (Tr1) cells, which block the activity of T-cells receptive to unidentified intestinal antigens, appears to be necessary for the immunosuppressive effects of IL-10 (the so-called antigen-driven bystander suppression) (23).This result was in agreement with (24, 25); who found that CD4 T cell population that produces IL-10 after recall is created in mice by administering Ag alone, and IL-10 production is increased during vaccination by adding LPS. This study finding is in agreement with Ye *et.al*, (26) who estimated that patients with infections from Gram-negative bacteria had dramatically raised levels of IL-10. Evaluation of the role of inflammatory cytokines in differentiating infection from Gram-positive from Gram-negative bacteria employed the detection of cytokines, such as IL-2 and IL-10. Some CD4 T cells regulate immune responses by inhibiting immune cells, these regulatory populations either develop in the thymus and constitutively express high levels of CD25, or differentiate in peripheral tissues and secrete IL-10; in addition to effector differentiation, T cell activation causes their proliferation, which is important for amplifying Ag-specific immune responses;

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Considering the central roles for Th cells in immunity, the effects of adjuvants on CD4 T cell accumulation and functional differentiation are of considerable interest (5).

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Conflict of interest

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

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