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Immunological and Haematological Study of Mice Immunized by *Serratia marcenes* Whole Cell Sonicated Antigen

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Abstract:

The study was designed to prepare antigens of sonicated *Serratia marcescens* and its effects on some interlukin (IL-8,IL-10) and Immunoglobulin-M (IgM)which were evaluated by using enzyme linked immunosorbant assay (ELISA)also, lymphocyte / neutrophil ratio at day 10,20,40 and 60 post immunization . For this purpose 24 mice randumly divided into four groups. The first group was immunized with killed whole cell sonicated *Serratia marcescens*(KWCSA-SM) (1000 µg/ml) Subcutaneously. Second group was immunized with killed whole cell sonicated Serratia marcescens(KWCSA-SM) (500 µg/ml) Subcutaneously .Third group was immunized with whole cell *Serratia marcescens* (1.5×10^8cfu/ml) ,4th group was was immunized by P.B.S.(pH7.2)as negative cotrole group. The high concentration of IL-8,IL-10 was recorded in the first group, It was (70%) respectively and the lowest concentration was in the fourth group(15%)respectively ,Also the results of IgM showed that the highest concentration was 65% in the first group while the lowest concentration was 13% in the fourth group.Lymphocyte/Neutrophil ratio showed a significant differences ($P<0.01$) between 2nd and 3rd groups also 5th group with significant differences ($P<0.05$).

Key words: *Serratia marcescens*, Antigens, Hematological Changes, IL-8,IL-10 , ELISA , Mice

INTRODUCTION

Serratia species are opportunistic gram-negative bacteria considered as opportunistic pathogen. *Serratia* are common in the environment, but are not a widespread component fecal flora of the human. [1]

Some strains of *S. marcescens* are capable of producing a pigment called prodigiosin. The chemical structure of prodigiosin has been unveiled, it was first used as marker in order to trace bacterial activity and transmission ,antibodies and T-cells can be triggered by this pigment .[2]

Inoculation of *S. marcescens* into the blood (hemolymph) of the Bombyx mori, silkworm induced caspase initiation and apoptosis of hemocytes. This procedure reduced the innate immune response in which pathogen cell wall contents, such as stimulate hemocytes ,glucan principal to the initiation of insect cytokine paralytic peptide. [3, 4].

Interleukin 8 (IL-8) is a chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells [3] and endothelial cells. Endothelial cells store IL-8 in their storage vesicles [5], the interleukin-8 protein is encoded by the *CXCL8* gene [6]. IL-8 is initially produced as a precursor peptide of 99 amino acids which then undergoes cleavage to create several active IL-8 isoforms.[7] In culture, a 72 amino acid peptide is the major form secreted by macrophages.[8].

(IL-10) is a protein that inhibits the synthesis of a number of cytokines, including IFN-gamma, IL-2, IL-3, TNF, and GM-CSF produced by activated macrophages and by helper T cells. In structure, IL-10 is a protein of about 160 amino acids that contains four conserved cysteines involved in disulphide bonds. IL-10 is highly similar to the *Human herpesvirus 4* (Epstein-Barr virus) BCRF1 protein, which inhibits the synthesis of gamma-interferon [9].

The innate immune response is characterized by the induction of pro-inflammatory cytokines, as well as increases in other accessory proteins that facilitate host recognition and elimination of the pathogens. The objective of the current study was to characterize the innate immune response during clinical mastitis elicited by *Serratia marcescens*,[10]. the pro-inflammatory cytokine response and changes in the levels of the innate immune accessory recognition proteins, soluble CD14 (sCD14) and lipopolysaccharide (LPS)-binding protein (LBP), were studied. Decreased milk output, induction of a febrile response, and increased acute phase synthesis of LBP were all characteristic of the systemic response to intramammary infection with either organism. Infection with this bacteria similarly resulted in increased milk levels of IL-1 beta, IL-8, IL-10, IL-12, IFN-gamma, TNF-alpha, sCD14, LBP, and the complement component, C5a. However, the duration of and/or maximal changes in the increased levels of these inflammatory markers were significantly different for several of the inflammatory parameters assayed. In particular, S. uberis infection was characterized by the sustained elevation of higher milk levels of IL-1 beta, IL-10, IL-12, IFN-gamma, and C5a, relative to *S. marcescens* infection. Together, these data demonstrate the variability of the innate immune response to two distinct mastitis pathogens [11].

Host recognition pathways for gram-negative and gram-positive bacteria comprise pattern recognition receptors among which Toll-like receptors (TLRs) play a pivotal role. TLRs share common signaling pathways yet exhibit specificity as well. Periodontal disease is initiated and maintained in the first line by gram-negative but also gram-positive bacterial infection of the gingival sulcus. To date only limited information is available on whether gram-positive and gram-negative bacteria induce different host responses (strength or quality) [12].

MATERIAL AND METHODS

ELISA kits (IL-4, IL-6 and IgM) Biosource(USA).

Laboratory animals:

A total number 24 mice of both genders which obtained commercially , were adapted for 1 week before started experiment by reared in separated clean and disinfected

cages; they were fed on commercial assorted pellets and clean tap water.

METHODS:

Antigen Preparation:

Killed whole cell antigen of *Serratia marcescens* **(KWCA-SM):**

Serratia marcescens killed whole cell antigen was prepared according to Motive (1992) procedure:

1. Bacterial culture on nutrient agar was incubated at 37 ºC for 48 hours to harvest a dense culture of bacteria; the bacteria were examined microscopically after staining with Gram stain to confirm a complete morphology of bacteria.
2. The bacteria was harvested by using PBS (pH 7.2), washed three times by PBS (pH 7.2) and then precipitated by centrifugation at 3000 rpm / 20 minutes.
3. The precipitate was resuspended with formalinized 0.3% PBS solution (pH 7.2); then incubated at 37 ºC for 1-2 hours, after that kept in a refrigerator (4 ºC) over night.
4. The bacterial suspension was washed three times with PBS (pH 7.2) and then precipitated by centrifuge 3000 rpm / 20 minutes and then resuspended with PBS pH 7.2 according to McFarland tube (No. 3) (3x108 CFU/ml) to be used for immunization.

Killed whole cell sonicated antigen of *Serratia marcescens* **(KWCSA-SM):**

Prepared according to Motive (1992) procedure

1. Killed whole cell antigen suspension of *Serratia marcescens* was prepared as in 1.2.3.4.
2. The suspension was sonicated by ultrasonicator at 15 KHZ/sec. rate for 30 minutes intervals in a cold environment.
3. The sonicated suspension of bacteria was centrifuged at 3000 rpm / 20 minutes; the supernatant was filtered by millipore filter (0.45µ), subsequent culture on Nutrient agar and blood agar and was microscopically examined to confirm a complete sonication and detected contamination. The suspension was kept frozen (-20Cº) till estimating the protein concentration.

Detection of protein concentration:

The protein concentration of *Serratia marcescens* was measured by using Biuret method according to[13].

Laboratory animal (mice) immunization:

Twenty four mice of both sexes were used which were randomly divided into five equal groups, as follows:

1. The first group was immunized with 1 ml (1000µg/ml) of KWCSA-SM subcutaneously.
2. The second group was immunized with 1ml (500µg/ml) of KWCSA-SM subcutaneously.
3. The third group (positive control group) was injected with 1 ml of (1000µg/ml) of KWCSA-SM subcutaneously.
4. The fourth group (negative control group) was immunized with 1 ml PBS (PH 7.2)subcutaneously.
5. At day 10 of immunization blood samples were collected from the direct puncture of the heart by sterile syringes for blood picture and sera were separated for estimate interlukins (IL-8,IL-10)and IgM concentration by ELISAkits .
6. At day 20, 40, 60, blood samples (1ml) were collected from all animal groups for Lymphocyte/Neutrophil ratio and to estimate the interlukines (IL-4,IL-6)and IgM concentration by ELISA.

Blood samples:

Blood samples (3 ml) were collected from the heart puncture of all animals at day 10, 20, 30, 40, 50, post immunization. Blood collected then kept in a slant position for few minutes until the clot formation and then separated by centrifuge at 3500 rpm for 10 minutes and the serum stored in a deep freeze (-20 ºC) according to [14].

Preparation of blood smears:

It was prerared according to [14].

1. A fresh (non-heparinized) sample of blood was added to one side of the slide.
2. The edge of another slide was pushed against the drop of blood and smeared onto the rest of the slide.
3. The blood film was fixed with methyl alcohol for 2 minutes in order to stabilize cellular components.
4. Pour Giemsa stain was diluted 1:9 with buffer over the smear for 8 -10 minutes.
5. Washed off with buffer and dry.
6. The dry and stained film was examined without a cover slip under oil immersion objective.
7. A total of 100 cells were counted in which every white cell seen was recorded in a table under the following heading: Lymphocyte, Neutrophil, to estimate Lymphocyte/neutrophil ratio.

Enzyme-linked immunosorbent assay (ELISA): ELISA kit*

Test principles

This ELISA kit uses Sandwich – ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to IL-8,IL-10. Standards or samples are added to the appropriate micro ELISA plate wells as combined with the specific antibody. Then a biotinylated detection antibody specific for IL-8,IL-10and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain IL- IL-8,IL-10, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2nm. The OD value is proportional to the concentration of IL- IL-8,IL-10. You can calculate the concentration of IL-8,IL-10in the samples by comparing the OD of the samples to the standard curve.

Materials provided:

Table(1):items of ELISA kit.

Item	Specifications	Storage
Micro ELISA plate	8 wells ×12 strips	4C
Reference Standard	2 vials	4C
Reference Standard and Sample Dilute	1 vial 20mL	4C
Concentrated Biotinylated Detection Ab	1 vial 120µL	4C
Biotinylated Detection Ab Diluent	1 vial 10mL	4C
Concentrated HRP Conjugate	1 vial 120µL	4C (shading light)
HRP Conjugate Diluent	1 vial 10mL	4C
Concentrated Wash Buffer (25×)	1 vial 30 mL	4C
Substrate Reagent	1 vial 10 mL	4C (shading light)
Stop Solution	1 vial 10 mL	4C
Plate Sealer	5 spieces	
Manual	1 copy	
Certificate of Analysis	1 copy	

* IL-8,IL-10 , (interleukin- IL-8,IL-10),Mybiosourse .USA.

Assay procedure:

1. Add 100µL standard or sample to each well. Incubate 90 minutes at 37C.
2. Remove the liquid. Add 100µLBiotinylated Detection Ab. Incubate 1 hour at 37C.
3. Aspirate and wash 3 times.
4. Add 100µL HRP Conjugate. Incubate 30 minutes at 37C.
5. Aspirate and wash 5 times.
6. Add 90µL Substrate reagent. Incubate 15 minutes at 37C.
7. Add 50µLStop solution. Read at 450 nm immediately.
8. Calculation of results.

Statistical analysis:

Analysis of results was done by using two ways of classification with interaction method and program of [15] SAS, (2010); detected the significant differences of this interaction. Two-way ANOVA was used and least significant differences (LSD) post hoc test was performed to assess significant differences among means. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Bacterial isolation results of one hundred and fifty milk tasters presented that 6 samples were give positive result to *S.marcescens* represented 4% from milk samples, [16].showed that 4(3%) out of 120 cow affected by *S. marcescens* mastitis and these outcomes in contract with the present study.

Bacterial culturing revealed different morphological features of bacteria on diverse media, after incubation at 37 °C for 24hours. On MacConkey agar colonies are lactose fermentor and appear red duo to the capability of *S. marcescens* to give red pigment as showed in Fig. (1) These marks as same with [17] isolated bacteria were showed under microscopic gram negative rods.

The Biochemical ID of *S. marcescens* showed that bacteria were Gram –ve, Rod, Catalase positive, oxidase negative, lactose non- fermenter, motile, Indole negative, citrate utilization positive, TSI y/y, DNase positive and Urease negative as [18] .To approve the diagnosis, RapID^{TM}ONE System and Api 20 E system.

The innate immune system initiates host defence against invasive microbial pathogens using specific recognition mechanisms. Here we review the current concepts and the molecular basis of innate immune responses to bacterial infections, focusing our attention on the actors involved in the response to Gram-negative bacteria. Lipopolysaccharide (LPS) is the major virulence factor of Gram-negative bacteria. During the past decade, enormous progress has been obtained in the elucidation of LPS recognition and signalling in mammalian phagocytes [19]**.**

Bacterial lipopolysaccharides (LPS) are the major outer surface membrane components present in almost all Gram-negative bacteria and act as extremely strong stimulators of innate or natural immunity in diverse eukaryotic species ranging from insects to humans. LPS consist of a poly- or oligosaccharide region that is anchored in the outer bacterial membrane by a specific carbohydrate lipid moiety termed lipid A. The lipid A component is the primary immunostimulatory centre of LPS. With respect to immunoactivation in mammalian systems[20]**.**

The normal gastrointestinal bacterial flora is crucial for the maturation of acquired immunity via effects on antigen-presenting cells (APCs). Here we investigated how two types of APCs, monocytes and dendritic cells (DCs), react to different bacterial strains typical of the commensal intestinal microflora. Monocytes produced higher levels of interleukin 12p70 (IL-12p70) , tumor necrosis factor (TNF), as detected by an enzyme-linked immunosorbent assay in response to gram negative bacteria. In contrast, DCs secreted large amounts of IL-12p70, TNF, IL-6, and IL-10 in response to gram negative bacteria. The lack of a response to the gram-positive strains correlated with lower surface expression of Toll-like receptor 2 (TLR2) on DCs than on monocytes[21] .

The normal gastrointestinal flora is in close and continuous contact with immune cells, and the resulting stimulation is essential for maturation of the immune system [22].

Antigen-presenting cells (APCs), such as monocytes, macrophages, and dendritic cells (DCs), are responsible for detecting microbes and presenting their antigenic structures to T cells, thus eliciting acquired immune responses[23]**.**

Table (2): Differentiation ratio of neutrophil between groups

Group	1	2		
A	A48.83±2.66ab	A40.83±3.87bc	A42.16±2.35b	B15.66±3.59b
B	AB41.00±3.67b	A49.83±3.28ab	B37.50±1.52b	C28.00±4.55a
C	A46.00±1.86b	A44.33±3.91ab	A52.00±2.73a	B23.50±2.14ab
D	A42.33±5.32b	A34.33±2.10c	A42.33±2.53b	B18.50±2.84b
E	A54.66±4.11a	A50.33±4.80a	A52.66±2.75a	A16.33±2.04b
LSD	9.2603			

Means with different small letter in the same column significantly different (P<0.05)
Means with different capital letter in the same row significantly different (P<0.05)

Table(3): Differentiation ratio of lymphocyte between groups

Group	1	2	3	4
A	B43.00±2.44ab	AB49.50±4.37ab	B47.33±1.99ab	A68.33±3.59a
B	A52.33±3.37a	B42.16±2.90b	A53.00±0.89a	A52.16±5.10bc
C	B44.83±1.93ab	B47.00±3.64ab	B42.16±1.64ab	A57.16±3.39b
D	AB52.66±4.65a	AB56.38±2.00a	B47.33±2.82ab	A59.16±4.79b
E	A36.33±3.48b	A42.83±3.87b	A39.16±2.56b	A45.83±4.22c
LSD	9.9725			

Means with different small letter in the same column significantly different (P<0.05)
Means with different capital letter in the same row significantly different (P<0.05)

Table(4): Differentiation ratio of Monocyte between groups

Group	1	2	3	4
A	BC6.33±1.28ab	AB8.00±0.89a	A9.66±0.95a	C4.00±1.03a
B	B5.50±1.40ab	AB6.33±1.11a	A9.16±0.98a	B6.16±1.04a
C	A8.00±0.25a	AB7.83±1.19a	B5.00±0.93b	B5.00±0.85a
D	B4.83±0.70b	A8.16±1.40a	A9.50±0.76a	B4.00±0.81a
E	AB7.00±0.89ab	AB6.00±1.09a	A7.83±0.87ab	B4.66±1.22a
LSD	2.8598			

Means with different small letter in the same column significantly different (P<0.05)
Means with different capital letter in the same row significantly different (P<0.05)

Table(5): Differentiation ratio of Basophil between groups

Group/B	1	2	3	4
A	B1.66±0.42ab	B.66±0.61a	B0.83±0.30a	A9.83±3.77b
B	B0.66±0.49b	B1.50±0.22a	B0.33±0.33a	A11.00±2.48b
C	B1.00±0.44ab	B0.83±0.54a	B0.83±0.65a	A9.66±1.76b
D	B4.83±0.70a	C0.50±0.34a	BC0.83±0.47a	A13.00±2.44ab
E	B2.00±0.63ab	B0.83±0.30a	B0.33±0.21a	A16.00±2.98a
LSD	4.0392			

Means with different small letter in the same column significantly different (P<0.05)
Means with different capital letter in the same row significantly different (P<0.05)

Table(6): Differentiation ratio of Eosinophil between groups

Group/E	1	2	3	4
A	B0.16±0.16a	B0.00±0.00a	B0.00±0.00a	A1.83±1.04ab
B	A0.00±0.00a	A0.16±0.16a	A0.00±0.00a	A0.83±0.47cd
C	B0.16±0.16a	B0.00±0.00a	B0.00±0.00a	A1.00±0.51bc
D	A0.00±0.00a	A0.00±0.00a	A0.00±0.00a	A0.00±0.00d
E	B0.00±0.00a	B0.00±0.00a	B0.00±0.00a	A2.33±0.76a
LSD	0.9411			

Means with different small letter in the same column significantly different (P<0.05)
Means with different capital letter in the same row significantly different (P<0.05)

We also analyzed the production of TNF, IL-6, and IL-10 from monocytes and DCs after bacterial stimulation**,** the gram-negative strains tended to induce higher levels of IL-6 and IL-10 from monocytes than the gram-positive bacteria [24].

Furthermore, macrophages differentiated in vitro from monocytes produced higher levels of TNF, IL-6, and IL-10 in response to gram-negative bacterial strains than they produced in response to gram-positive bacterial strains, even though the difference was not as pronounced as that

observed with DCs). In conclusion, while monocytes responded to both the gram-positive and gram-negative strains, albeit with different cytokine patterns, DCs produced high levels of cytokines only in response to the gram-negative bacteria [25].

The difference in the capacities of monocytes and DCs to respond to the different bacterial strains might be due to differences in phagocytic capacity [26]**.** Whether monocyte-derived DCs represent the DCs located in the gut is questionable. However, in line with our results, experiments with intestinal mucosal explants have shown that TNF, IL-8, and IL-10 are produced in response to *Serratia marcescens* infection [27]**.**

Table(7): Differentiation ratio of IL8 **between groups**

Group/IL8	1	2	3	4
A	0.361±0.004a	0.363±0.004	0.362±0.003	0.361±0.008
B	0.363±0.007a	0.359±0.002	0.359±0.003	0.359±0.008
C	B0.318±0.03b	A0.360±0.01	A0.367±0.005	A0.376±0.01
D	0.356±0.002a	0.377±0.008	0.365±0.003	0.354±0.003
E	0.355±0.002a	0.357±0.003	0.356±0.005	0.383±0.02
LSD	0.0315			

Means with different small letter in the same column significantly different (P<0.05)
Means with different capital letter in the same row significantly different (P<0.05)

Table(8): Differentiation ratio of IL10 **between groups**

Group/IL10	1	2	3	4
A	A0.389±0.004a	A0.367±0.005b	A0.371±0.008a	A0.395±0.01a
B	A0.399±0.01a	A0.358±0.005b	A0.401±0.01a	A0.378±0.01a
C	A0.401±0.02a	A0.372±0.01b	A0.370±0.005a	A0.379±0.01a
D	A 0.389±0.01a	A0.386±0.01ab	A0.395±0.02a	A0.391±0.01a
E	B0.371±0.006a	A0.422±0.03a	B0.363±0.004a	AB0.392±0.01a
LSD	0.0418			

Means with different small letter in the same column significantly different (P<0.05)
Means with different capital letter in the same row significantly different (P<0.05)

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