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Immune response of rabbits immunized by killed whole cell sonicated *E. coli* 0157 antigen and crude Klebocin

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Abstract
This present research was aimed to estimate the immune responses of rabbits immunized by KWCSA-*E.coli* 0157 and Klebocin during the period from November-2016 to May-2017. Thirty rabbits of both genders were randomly divided into six equal groups (5 animals for each group). Three types of tests were performed which were Skin Test (The delayed type hypersensitivity (DTH)), Tube Agglutination Test and ELISA. The Skin Test was performed at 18 days post immunization. The results showed an elevation in the means of erythema at 24hr of the 1st, 2nd, 3rd,4th and 5th groups which were 9.70, 8.90, 8.20, 6.00± 0.15 and 5.60 mm respectively. There was also a significant increasing (P<0.05) in the diameter of induration of the same sequence groups (8.30, 8.00, 7.60, 5.00 and 4.50 mm). The results of tube agglutination test showed at 20 days the highest titration was (1120.00) in the first group, while the lowest titer was 160.00 in the fifth group with significant differences (P<0.05). On the other hand ELISA showed elevation at 20 days in both of IL-2 (180.82) and IL-6 (107.69) levels in first group whereas the lowest concentration at 60 days post immunization in all groups, with significant differences (P<0.0 5). The results of the present study indicated that there was an interaction between both antigens which could enhance the humoral and cellular immune response.

Keywords: Enter-hemorrhagic *Escherichia coli* O157, Klebocin, DTH, TAT

1. Introduction

Escherichia coli are an important zoonotic agents transmitted from animal to human especially veterinarian and who lived with these animals in the same place though direct contact [7]. Entero-hemorrhagic *E.coli* (EHEC) serotypes O157, which are expresses somatic (O) antigen and flagella (H) antigen, caused serious morbidity and large disease outbreaks leading to become this bacterium one of the most important food-borne and waterborne pathogens worldwide and also because production potent toxins (Shiga toxin) [37, 16, 2]. They caused severe damage in the intestine and it's necessary for induction of entero-pathogenic and haemolytic-uraemic [36]. They also cause illness through food, which cause hemorrhagic diarrhea, and the post diarrheal hemolytic uremic syndrome (HUS) that leading to kidney failure [8].
The humoral immunity plays a great role in the adaptive immune response against *E. coli* O157:H7 [41]. After engulfment, a particular antigenic sequence is presented on the MHC molecules (class 2) of antigen presenting cells which then activate CD4+ T helper cells.These activated anther T cells then go on to activate naive B lymphocytes which undergo clonal expansion and differentiate into antibody producing plasma cells and memory B cells specific for the antigenic sequence[27]. When secondary exposure to the bacterium is happened, the memory B cells are capable to converting for plasma cells, which secreting antibodies and mounting a humoral response quicker and greater than before. [15]. The most common type of antibodies are IgA antibodies, which found in abundance in the lumen of the gastrointestinal tract[44], These antibodies are recognition of a specific antigenic to attachment and formation of an antibody-antigen complex[29]. That complex activates the classical pathway of the complement system then allows for recruitment of inflammatory cells, opsonization and direct killing of pathogens by lysis [30]. Cytokines are identified in many ways that include the numeric order of discovery and they can be identified by their kinetic role in the inflammation (pro – inflammatory cytokine) [6]. IL2 and IL6 evoke strong antibody responses and eosinophils accumulation nevertheless inhibit several functions of phagocytic cells [33].Cytokines are not only important for amplifying the host defense against invading microbes, but to modulate the intensity of innate immunity and to polarize and transition the

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innate immunity to adaptive immunity [38].

In addition the cytokines such as interferon gamma (IFN$_\gamma$), Tumer Necrosis Factor alpha (TNFα) interferon-inducible protein 10 (IP-10) and interleukins (IL7, IL6 and IL12) have an important role during pulmonary *k. pneumonia* infection as they increased mortality and bacterial burden in lungs [20, 25]. Klebocin or Klebcin as closed circle with molecular weight 5-220 kDa produced in different amounts from *K. pneumonia* species, it was introduced to designate toxic proteins produced by a given strain of Klebsiella species and active against related species cells [10].

That required cloacin DF13 receptor that found in *E.coli* O157 has an important immunological role between klebocin and *E.coli* O157 interaction [4, 34].

The objective of the present study was aimed to evaluate the immune response of rabbits immunized by killed whole cell sonicated *E. coli* 0157 antigen and crude Klebocin and to investigate the effect of both antigens on the humoral and cellular immune response.

2. Materials and Methods
This present research was conducted to estimate the immune responses of rabbits immunized by KWCSA-*E.coli* 0157 and Klebocin during the period from November-2016 to May-2017.

2.1 Bacterial isolate
E.coli O157 and *klebsiella pneumonia,* was obtained from pathology Unit/ College of Veterinary Medicine/ Baghdad University.

2.2 Antigens Preparation
A-Killed whole cell sonicated *E.coli 0157* antigen *(KWCEA)* Prepared according to [43].
B-Klebocin was prepared according to Chhibber and Vadehra [3].
C-The protein concentration of *E.coli 157 and Klebocin* was measured by Biuret method according to Henry *et al*. [13].

2.3 Laboratory animal (rabbits) immunization
Thirty rabbits of both gender which were randomly divided into six equal groups (5 animals for each group), as follows: The first group was immunized with (1000μg/ml) of KWCSA-*E.coli* 157 and (1000μg/ml) of Klebocin. Subcutaneously. The second group was immunized with (1000μg/ml) of KWCSA-*E.coli 157 and* (500μg/ml of Klebocin subcutaneously. The third group was immunized with KWCSA- *E.coli* 157, (1000μg/ml) antigen subcutaneously. The forth group was immunized with (1000μg/ml) of Klebocin subcutaneously. The fifth group was immunized with (500μg/ml) of klebicin subcutaneously and the sixth group (negative control group) was injected with 1ml PBS (PH 7.2) subcutaneously.

2.4 Blood samples
Blood samples (3 ml) were collected from the heart puncture of all animals at day 10, 20, 40, 60, post immunization and the serum stored in a deep freeze (-20 ºC) according to [42].

2.5 Immunological tests
A- Delayed type hypersensitivity (DTH) skin test this test according - to [14].
B-The tube agglutination test (TAT) according to [11].
C-Enzyme-linked immunosorbent assay (ELISA) kit Rabbit IL-2, IL6, cusabio (china kit).

Statistical analysis: The statistical analysis was performed using SAS (Statistical Analysis System - version 9.1) [35].

3. Results
3.1 Delayed type hypersensitivity test (DTH) Skin Test
The results of skin erythema showed that there was a significant (P<0.05) elevation in the mean diameter of erythema at 24hr in all treatments as shown Tables 1, 2, 3, 4, and 5. The highest mean (9.70 mm) was found in the group of the rabbits immunized by KWCSA-*E.coli* 0157 and klebocin (1000μg/ml)(Table 1) while the lowest (5.60 mm)was found in the group of the rabbits immunized by Klebocin (500μg/ml) (Table 5). In general all diameters were decreased significantly (P<0.05) along with period (48 and 72 hrs) and dilutions (diluted 1:2 and 1:4) in all treatments. Concerning the means of induration, it was shown that a significant (P<0.05) increasing in the means at 24 hrs in all treatments as illustrated in the Tables 6, 7, 8, 9, and 10. The trend of response in the means of duration is similar to that in erythema test as the means significantly decreased (P<0.05) across the period and dilutions.

Table 1: Means of skin erythema (mm) of all rabbits immunized by KWCSA-*E.coli* 0157 and klebocin (1000μg/ml).

Time / Concen.	Means+ S.E(m.m)		
	24 hrs.	48 hrs.	72 hrs.
Concentrated antigen	9.70±0.25 A a	8.00±0.27 B a	6.50±0.22 C a
Diluted 1:2	7.60±0.24 A b	6.00±0.15 B b	5.00±0.15 C b
Diluted 1:4	6.80±0.12 A c	5.60±0.24 B c	4.10±0.10 C c
Control region	1.30±0.12 A d	0.80±0.12 AB d	0.50±0.00 B d

Table 2: Means of skin erythema (mm) of all rabbits immunized by KWCSA-*E.coli* 0157(1000μg/ml) and Klebocin (500μg/ml).

Time / Concen.	Means+ S.E(m.m)		
	24 hrs.	48 hrs.	72 hrs.
Concentrated antigen	8.90±0.24 A a	7.40±0.24 B a	6.20±0.12 C a
Diluted 1:2	7.00±0.27 A b	5.90±0.10 B b	4.30±0.21 C b
Diluted 1:4	6.20±0.12 A c	5.40±0.24 B b	4.20±0.20 C b
Control region	1.20±0.12 A d	0.70±0.12 AB c	0.44±0.06 B c

Table 3: Means of skin erythema (mm) of all rabbits immunized by KWCSA-*E.coli*0157(1000μg/ml).

Time / Concen.	Means+ S.E(m.m)		
	24 hrs.	48 hrs.	72 hrs.
Concentration antigen	8.20±0.12 A a	7.00±0.40 B a	6.10±0.20 C a
Dilution 1:2	6.80±0.20 A b	5.80±0.12 B b	4.00±0.25 C b
Dilution 1:4	5.90±0.10 A c	4.80±0.20 B c	4.00±0.03 C b
Control region	1.00±0.15 A d	0.70±0.12 AB d	0.40±0.06 B c

Table 4: Means of skin erythema (mm) of all rabbits immunized by (Klebocin 1000µg/ml)

Time Concen.	Means+ S.E(m.m)		
	24 hrs.	48 hrs.	72 hrs.
Concentrated antigen	6.00±0.15 A a	5.10±0.10 B a	4.20±0.12 C a
Diluted 1:2	4.70±0.12 A b	3.50±0.22 B b	2.60±0.10 C b
Diluted 1:4	3.90±0.24 A c	3.40±0.24 B b	2.50±0.15 C b
Control regain	0.60±0.10 A d	0.50±0.03 A c	0.20±0.06 A c

Table 5: Means of skin erythema (mm) of all rabbits immunized by Klebocin (500µg/ml).

Time Concen.	Means+ S.E(m.m)		
	24 hrs.	48 hrs.	72 hrs.
Concentrated antigen	5.60±0.24 A a	4.70±0.20 B a	3.90±0.24 C a
Diluted 1:2	4.10±0.10 A b	3.00±0.05 B b	2.60±0.10 C b
Diluted 1:4	3.40±0.20 A c	2.90±0.18 B b	2.20±0.20 C b
Control regain	0.60±0.10 A d	0.50±0.00 A c	0.20±0.06 A c

Table 6: Means of skin induration of all rabbits immunized rabbits by KWCS-*E.coli* 0157 and klebocin (1000µg/ml).

Time Concen.	Means+ S.E(m.m)		
	24 hrs.	48 hrs.	72 hrs.
Concentrated antigen	6.20±0.12 B a	7.90±0.10 A a	8.30±0.20 A a
Diluted 1.2	4.30±0.20 C b	5.70±0.20 B b	6.40±0.18 A b
Diluted 1.4	3.90±0.10 B b	4.80±0.20 A c	5.10±0.10 A c
Control regain	1.30±0.12 A c	0.80±0.12 AB d	0.60±0.12 B d

Table 7: Means of skin induration of rabbits immunized by KWCS-*E.coli* 0157(1000µg/ml) and klebocin (500µg/ml).

Time Concen.	Means+ S.E(m.m)		
	24 hrs.	48 hrs.	72 hrs.
Concentration antigen	5.70±0.20 C a	7.30±0.12 B a	8.00±0.05 A a
Diluted 1:2	4.00±0.05 C b	5.20±0.12 B b	6.10±0.10 A b
Diluted 1:4	3.70±0.20 C b	4.40±0.24 B c	5.10±0.18 A c
Control regain	1.00±0.15 A c	0.70±0.12 AB d	0.50±0.10 B d

Table 8: Means of skin induration of rabbits immunized by KWCS-*E.coli* 0157 (1000µg/ml).

Time Concen.	Means+ S.E(m.m)		
	24 hrs.	48 hrs.	72 hrs.
Concentrated antigen	5.40±0.18 C a	6.90±0.10 B a	7.60±0.18 A a
Diluted 1.2	3.80±0.12 C b	4.60±0.24 B b	5.30±0.20 A b
Diluted 1.4	3.00±0.36 C b	4.20±0.12 B b	5.00±0.20 A b
Control regain	0.90±0.10 A c	0.60±0.10 C c	0.50±0.05 B b

Table 9: Means of skin induration (mm) of all rabbits immunized by Klebocin (1000µg/ml).

Time Concen.	Means+ S.E(m.m)		
	24 hrs.	48 hrs.	72 hrs.
Concentrated antigen	3.50±0.15 C a	4.70±0.20 B a	5.00±0.20 A a
Diluted 1:2	3.20±0.12 C ba	3.80±0.12 B b	4.30±0.12 A b
Diluted 1:4	2.30±0.12 B b	2.60±0.18 B c	3.40±0.24 A c
Control regain	0.80±0.12 A c	0.50±0.00 AB d	0.38±0.07 B d

Table 10: Means of skin induration (mm) of all rabbits immunized Klebocin (500µg/ml).

Time Concen.	Means+ S.E(m.m)		
	24 hrs.	48 hrs.	72 hrs.
Concentrated antigen	3.00±0.10 C a	4.10±0.18 B a	4.50±0.22 A a
Diluted 1:2	2.90±0.10 C a	3.60±0.24 B b	4.10±0.09 A b
Diluted 1:4	1.90±0.10 C b	2.40±0.10 B c	3.00±0.36 A c
Control regain	0.80±0.12 A c	0.50±0.10 AB d	0.38±0.07 B d

In all tables of skin test:
-Means with different capital letters in the same row significantly different (P<0.05).
-Means with different small letters in the same column differ significantly (P<0.05).
The highest mean of duration (6.20) was detected in the rabbits immunized rabbits by KWCS-*E.coli* 0157 and klebocin (1000µg/ml) whereas the lowest (3.00) was noticed in the rabbits immunized by Klebocin (500µg/ml).

3.2. Tube Agglutination Test (TAT)
The results of the tube agglutination test showed that the differences were significant (P<0.05) among groups within all periods (10, 20, 40, 60 days). The mean of all groups significantly increased (P< 0.05) with advanced period. The means of all groups reached the peak at 20 days. The mean of the group KWCSA-*E.coli*157 and klebocin 1000µg/ml (1120) at the 20 days was significantly (P<0.05) accessed the means of all other groups. All groups showed a gradual decreasing in the means at 40 and 60 days.

Table 11: Means of tube agglutination test of all groups immunized rabbits by WCS-*E.coli* 157 and klebocin.

days / Groups	Means+ S.E(m.m) tube agglutination test titration-			
	10 days	20 days	40 days	60 days
KWCSA-*E.coli*157 and klebocin 1000μg/ml	560.00±80.00 B C a	1120.00±160.00 Aa	800.00±160.00 B a	475.00±89.58 C a
KWCSA-*E.coli*157 and klebocin 500μg/ml	480.00±92.37 B a	960.00±184.75 A b	640.00±0.60 B a	395.00±75.00 B a
KWCSA-*E.coli*157 1000μg/ml	440.00±120.00 B a	880.00±240.00 A b	560.00±80.00 B a	320.00±30.10 B ab
klebocin 1000μg/ml	180.00±50.33 A b	200.00±40.00 A c	160.00±25.00 A b	100.00±20.00 A bc
klebocin 500μg/ml	100.00±20.00 A b	160.00±25.00 A c	140.00±20.00 A b	80.00±0.50 A c
Control	10.00±5.77 A b	17.50±5.00 A c	12.50±5.00 A b	15.00±5.00 A c

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

3.3. Enzyme linked immunosorbent assay (ELISA)

Both IL-2 and IL-6 showed a significant (P< 0.05) elevation in the first group, which was 180.82 and 107.69 respectively and the lowest concentration, at 60 days post immunization in all groups with significant differences P<0.0 5) Table (12) and (13).

Table 12: IL-6 concentration in the immunized rabbits immunized with different antigens.

Time / Groups	Means± S.E- IL-6concentration			
	10 days.	20 days.	40 days.	60 days.
KWCSA-*E.coli*157 and klebocin 1000μg/ml	96.86±16.54 B a	107.69±23.57 A b a	88.81±4.74 B cd	67.72±15.01 B a
KWCSA-*E.coli*157 and klebocin 500μg/ml	78.18±18.58 AB a	90.57±22.15 A a	75.76±8.57 B b	61.43±23.83 AB a
KWCSA-*E.coli*157 1000μg/ml	71.33±15.97 A a	83.44±19.89 A ab	70.51±9.45 A ab	55.76±7.36 A a
klebocin 1000μg/ml	94.93±15.90 A a	112.56±14.78 A a	110.50±9.28 A a	74.79±5.02 A a
klebocin 500μg/ml	82.63±12.09 A a	90.10±19.89 A ab	85.37±1.85 A ab	65.79±21.43 A a
PBS	49.64±6.86 A a	52.97±11.81 A b	51.49±8.84 A b	54.82±15.72 A a

Table 13: IL-2 concentration in the immunized rabbits immunized with different antigens.

Time / Groups	Means± S.E- IL-2concentration			
	10 days.	20 days.	40 days.	60 days.
KWCSA-*E.coli*157 and klebocin 1000μg/ml	163.61±2.98 BC ba	180.82±3.18 AB a	193.14±3.68 A a	158.97±4.26 C a
KWCSA-*E.coli*157 and klebocin 500μg/ml	156.93±4.13 B a	175.19±1.62 A a	189.43±4.07 A a	155.68±6.59 B a
KWCSA-*E.coli*157 1000μg/ml	167.33±7.18 B a	181.69±6.70 A a	187.19±6.41 A a	141.51±8.48 C a
klebocin 1000μg/ml	126.63±7.95 A b	129.31±6.69 A b	110.37±11.75 A b	80.29 ±13.07 B b
klebocin 500μg/ml	115.18±9.72 AB bc	123.60±8.50 A b	107.84±6.63 AB b	94.97±5.02 B b
PBS	94.60±4.60 A c	89.65±9.35 A c	99.10±0.09 A b	92.04±2.00 A b

-Means with different small letters in the same column differ significantly (P<0.05)
-Means with different capital letters in the same row differ significantly (P<0.05).

4. Discussion

Skin test of all groups represented by the mean of skin erythema and induration reaction for each immunized group; that occurred due to the role of memory cell which modulate the Th-1 to secrete Interferon-γ (INF-γ), potent mediator that stimulates the migration of macrophage to the site of reacted area of skin [34]. while the macrophage secrete Interleukin-1 (IL-1) that enhance proliferation and differentiation of other T cells into T helper-1(Th-1) cells which secrete Interleukin-2 (IL-2) that consider a chemotactic factor, it cause attraction of macrophages around area of activated T cells [24]. The antigen presenting cells was firstly taking up the foreign protein and broke it into many peptides and then bound to the binding sit class II MHC molecule; this immunogenic peptide was recognized by T-cell antigen receptor, the T-cell induce the helper activity secretion of lymphokine (CD4+) that recognize the (antigen–MHC II complex), then developed into cytotoxic cells (CD8+) which recognize the class I MHC molecule. When the skin of the sensitized animal injected with the certain antigen, an inflammatory response occur taking many hours to develop the action on injected site [40]. Our result agree with the studies which obvious that *E.coli* O157 antigen

are able to induce cellular immune response as detected by DTH-skin test immunization animal whose record a highly significant increasing of skin erythema in 24 hr and increase in induration after 72 hr [19]. The present study observation is consistent with another research that describe the Skin Test depend on ability and activity of Th-cells to recognize antigen and secrete IL-1, which enhanced proliferation and different-tiation of other T-cell into Th-cells, which secrete IL-2 as a chemoat-ractive factor to attract macrophage around the area of activated T-cell that also secrete INF- that enhancing the cytolysis activity of accumulated macrophages leading into skin thickness [34, 45].

The antibodies titer was elevated after 20 days of booster dose administration, in all immunized groups compared with control group, especially in the first group, which had the highest antibodies titer, our findings agree with Oladejo and Adebolu which showed the antibody titration agents E.coli O157 antigen as first dose elevated then reach to the peak after booster dose from post immunization [31]. Also, agreed with Karmali studies that showed the shiga-like toxin neutralization antibody produced different titration agents E.coli antigen [16].

CD4+ T-cell colonies were classified into the Th1 and Th2, Th1 involved in cell-mediated immune reaction it secreted IL-2 while Th2 is associated with strong antibody and allergic responses, and it secreted IL-6 [28]. Antigens of this microorganism cause immune response to stimulation pro-inflammatory cytokine such (IL2-&IL-6) in E.coli and in the k. pneumonia were elevated in all groups especially in first, second group due to LPS act as activated IL-6 production [22,12,21].our result agree with [18] which report that CD34+ stem cells and their cultured cells up-regulated expression of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor (TNF) after infection with E. Coli expression E. coli 0157 cause up-regulated expression of the pro-inflammatory Cytokines to elevated IL-6 that agree with this study. IL-6 is also elevated in all groups especially in the first, second, fourth groups because the production of pro-inflammatory cytokines and inhibit microbial activities of macrophages, neutrophils, neutralizing IL-6 in K. pneumoniae infected lab animals which deceased mortality and lung bacterial burden aiding the host in pulmonary clearance, that agree with Greenberger and another researcher, that showed the IL-6 is acritical mediator of survival k. pneumoniae infection and suggest that IL-6 protects from death by augmenting neutrophil killing of bacteria [9, 39].

Elevated of IL-2 all immunize group especially in first, second, third groups, Activated Th1 cells secrete more IL-2 and IFN-γ to activate macrophages for more active killing. IFN-γ also increased production of IL-12 by dendritic cells and macrophages when infected with E.coli O157 that agrees with this study. IL-2 may act as growth factors for E.coli then elevated IL-2 in serum [1, 5]. Kashima showed elevation in IL-2 and IL-6 in E.coli infection [17] and that agree with this study.

5. Conclusion
The results of the present study indicated that there was an interaction between both antigens which could enhance the humoral and cellular immune response.

6. Acknowledgement

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