Immune response interaction of *Klebsiella pneumoniae* and *Eimeria tenella*

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Summary

This study aimed to investigate the immune responses (Cellular and humoral) to *Klebsiella pneumoniae* and *Eimeria tenella* by using Delayed Type Hypersensitivity Test (Skin Test) and Tube Agglutination Test in sixteen local breed rabbits. Animals were divided into four equal groups; The first group was immunized subcutaneously with 1 ml (1000 µg/ml) of sonicated *Eimeria tenella* oocysts –SETO-ET), the second group was immunized subcutaneously with 1 ml (1000 µg/ml) of sonicated Whole *Cells Klebsiella pneumoniae* antigen –SKWC-KP), and the third group was immunized subcutaneously with 1 ml (500 µg/ml of sonicated *Eimeria tenella* –SETO-ET and 500 µg/ml SKWC-KP). The fourth control group was injected subcutaneously by 1 ml of phosphate buffer saline. After 14 days, all groups were given a booster dose at the same dose above. Results of Delayed type hypersensitivity showed that the third group had a high significant ($P \leq 0.05$) skin test reaction of Erythema and induration compared to the first and second groups after 24, 48 and 72 hours. Also, that was synchronized with the increased titers of antibodies, which increased to peak (720 ± 201.32) compared with the first group (200 ± 40.00) and second group (360 ± 100.66) after 35 days. This was the first study about the synergistic immune response interaction between *Klebsiella pneumoniae* and *Eimeria tenella* antigens in Iraq.

Keywords: *Klebsiella Pneumoniae*, *Eimeria tenella*, Skin test, Interaction.

Introduction

Klebsiella pneumoniae is a very common pathogen that causes several infections (urinary tract, nosocomial pneumonia and intra abdominal infections); abscess in liver, lungs and brain; pyogenic meningitis; septic arthritis; osteomyelitis and endophthalmitis (1). Infection causes necrosis, inflammation, and hemorrhage within the lung tissues caused by aspirating oropharyngeal microorganisms into the lower respiratory tract and causes meningitis with symptoms including sharp head pain, nausea, dizziness, and impaired memory. In recent years, *K. pneumoniae* has been associated with increased invasiveness and pathogenicity, which appears to be an important virulence factor as promotes evasion of innate immune defenses (2). *Klebsiella pneumoniae* escapes from the host defense by shuttering the rapid and prolonged immune activation by modifying its lipid A structure from the hexa-acylated (potent antagonist) to hepta-acylated form (weak antagonist). In addition, the increased number of acyl chain in fatty acids and the presence of palmitate, 4-aminoarabinose along with hepta-acylated lipid A (12 and 24 hr.) augmented the bacterial resistance against immune defense, which further supported the survival as well as multiplication of pathogen inside the host (3).

The complex immune response to *Eimeria* involves many facts of specific and non specific immunity, the latter encompassing of both cellular and humoral immune mechanisms (4). The cell mediated immunity is playing a major role in protection from *Eimeria* (5). The CD 4^+ T helper (Th) cells and $DC8^+$ Cytotoxic T-lymphocytes (CTLs) are the major T cell subsets involved in the host response to Eimeria infection (6). T-lymphocyte and their cytokines are essential for immunity against *E. tenella* infection in both avian and mammalian species (7). The macrophage inhibiting factors (MIF) induces the expression of proinflammatory mediators, including IL-1b, IL-6, IL-8, IFN-c and TNF-a (8). In this study, we aimed to estimate the immune response interactions between *Klebsiella pneumoniae* and *Eimeria tenella*.

Materials and Methods

Klebsiella pneumoniae was provided from the Zoonosis Diseases Unit/ College of Veterinary Medicine -Baghdad University. *Eimeria tenella* oocysts were isolated from infected broiler chicken seca (9). Killed Whole

Cell Sonicated *Klebsiella pneumoniae* Antigen (SKWCS - KP) was prepared according to (10) with some modification and used to immunize the rabbits and apply skin test (DTH). Sonicated *Eimeria tenella* oocysts antigen (SETO-ET). The oocysts of *Eimeria tenella* were collected from the infected seca of chicken according to (11); these oocysts were sonicated to prepare sonicated antigen according to (10). The protein of each antigen was measured by using Biuret method according to (12). Sixteen local breed rabbits of both sexes (1.5-2 Kg B.W.) were used, they were divided randomly into four equal groups: The first group was immunized subcutaneously with 1ml of 1000 µg/ml of sonicated *Eimeria tenella* oocysts (SETO.-ET). The second group was immunized subcutaneously with 1ml of 1000 µg/ml of *Klebsiella pneumoniae* – SKWC-KP. The third group was immunized subcutaneously with both 500 µg/ml of SKWC–KP and 500 µg/ml SETO-ET. The fourth control group was injected subcutaneously with 1ml of phosphate buffer saline –PBS (pH 7.2). Booster dose of the immunogens was given in the same dose to all groups after 14 days of first dose. Delayed Type Hypersensitivity (DTH)-Skin Test was applied to all immunized animals at day 20 post immunization as described by (13) with some modification; Different protein concentrations were used (Crude) of sonicated KWC antigen of *Klebsiella pneumoniae*; concentrated antigen (14 µg / ml). 1:2 dilution (7 µg / ml) antigen and 1: 4 dilution (3.5 µg / ml) antigen and PBS (pH 7.2) was injected as a control region, by intradermal route.

Blood samples were collected by heart puncture each 14 days for three intervals after immunization. Sera were separated and stored in deep freeze until used (14). Tube agglutination test was carried according to (15). One-way ANOVA was performed. Means were compared using least significant differences. P<0.05 considered significant.

Results and Discussion

Skin test (Delayed type Hypersensitivity-Erythema) initiated due to the crude *Klebsiella pneumonia* antigen showed a significant increase in thickness (P≤0.05) of all immunized groups (*E. tenella*, *K. pneumonia* and *E. tenella* and *K. pneumonia)* as compared with control group (Phosphate buffer saline) at 24, 48 and 72 hours. The group immunized with E. *tenella and Klebsiella* recorded the highest increase (8.21 ± 0.62mm) after 24 hours with a significant difference (P≤0.05) compared with the groups that were immunized with *E . tenella* alone or *E. tenella and Klebsiella* after 48 and 27 hours (Table, 1). Also significant differences (P≤ 0.05) were recorded between all immunized groups and control group when tested at half concentration of *K. pneumonia* antigen. A significant difference (P≤0.05) was found only between *K. pneumonia* immunized group and *E. tenella* after 48 and 72 hours (Table, 2), while a significant difference (P≤0.05) was recorded between these two groups after 24 and 48 hours when examined at 1/4 concentration of *K. pneumonia* antigen, but no significant difference (P≥0.05) between all immunized groups after 72 hrs. All immunized groups after 24, 48 and 72 hrs. with a significant difference (P≤0.05) compared with control group when examined by 1/4 concentration of *K. pneumonia* antigen. (Table, 3).

Table, 1: Diameters of erythema (Skin test) in different groups of immunized rabbits and tested by whole crude sonicated *K. pneumonia* antigen.

Group	Diameter of erythema (Means ±SE mm)		
	Hours		
	24	48	72
E.tenella	7.23±0.80 A	4.90±0.53 A	2.48±0.31 A
K.pneumonia	5.62±0.48 AB	4.98±0.60 A	3.61±0.51 A
E. tenella and *K.pneumonia*	8.21±0.62 AC	6.92±0.21 B	5.92±0.51 B
Phosphate buffer saline	1.75±0.47 D	1.25±0.40 C	0.75±0.25 C

Different capital letters means a significant difference between groups (P≤0.05).

The cutaneous reaction in the skin test (DTH)could be initiated by the activation of CD4 T cells that activated by antigen presenting cells in the skin releasing inflammatory mediators that recruit the macrophage (monocytes), CD8 cytolytic T cells and NK cells that elicit the inflammatory response to eliminate the antigen (16). That is explain our results in the increase the erythema and indurations when using of the both

antigens and in the *E. tenella* immunized groups, which *K. pneumoniae* escapes from the host defense by shuttering the rapid and prolonged immune activation by modifying its lipid A, which augmented the bacterial resistances against immune defense, and further supported the survival, as well as, multiplication of pathogen inside the host. Also, this bacteria attaches to the host to evade the immune defense of the host and cause the fatal infection (3). The mechanisms of protection, as the bacterium evade phagocytosis by polymorphonuclear granulocytes. This mechanism prevents killing by bactericidal serum factors and inhibits complement constituents, such as C_3b, which opsonize the pathogen. These effective virulence factors have the ability to inhibit the differentiation capacity of macrophages (2).

Table, 2: Diameters of erythema (Skin test) in rabbits immunized with sonicated *K. pneumonia* and *E. tenella* by 1/2 crude sonicated *K. pneumonia* antigen.

Group	Diameter of erythema (Means ±SE mm)		
	Hours		
	24	48	72
E.tenella	5.92±0.41 A	4.50±0.34 A	3.64±0.41 A
K.pneumonia	4.52±0.38 AB	3.62±0.27 AB	2.98±0.35 A
E.tenella and *K.pneumonia*	7.31±0.45 AC	5.48±0.61 AC	3.76±0.40 A
Phosphate buffer saline	1.25±0.25 D	1.00±0.40 D	0.75±0.25 B

Different capital letters mean a significant difference between groups (P≤0.050).

Table, 3: Diameters of erythema (Skin test) in rabbits immunized with sonicated *K. pneumonia* and *E. tenella* by 1/4 crude sonicated *K. pneumonia* antigen .

Group	Diameter of erythema (Means ± SE mm)		
	Hours		
	24	48	72
E.tenella	4.01±0.33 A	3.24±0.52 A	2.31±0.34 A
K.pneumonia	3.22±0.41 A	2.46±0.32 AB	1.92±0.22 A
E.tenella and *K.pneumonia*	5.41±0.30 B	3.48±0.30 AC	2.90±0.45 A
Phosphate buffer saline	1.00±0.00 C	0.50±0.28 D	0.02±0.25 B

Different capital letters means a significant difference between groups (P≤ 0.05).

In induration, also the immunized group by *E. tenella* and *K. pneumonia* was a high diameters than other immunized groups (*E. tenella* and *E. tenella* and *K. pneumonia*) with a significant difference (P≤0.05) compared with the control group when examined by crude concentration of *K. pneumonia* antigen (Table, 4), but only a significant difference (P≤0.05) was found between the *K. pneumonia* and *E. tenella* and *K. pneumonia* after 24 hrs. only when examined by 1/2 concentration of *K. pneumonia* antigen (Table, 5). There was no significant difference (P≥0.05), as shown in (Table, 6).

Table, 4: Diameters of induration (Skin test) in rabbits immunized with sonicated. *K. pneumonia* and *E. tenella* by crude sonicated *K. pneumonia* antigen.

Group	Diameter of erythema (Means±SE mm)		
	Hours		
	24	48	72
E.tenella	5.13±0.51 A	6.81±0.41 A	7.23±0.43 A
K.pneumonia	4.10±0.51 A	5.63±0.71 A	6.73±0.41 A
E.tenella and *K.pneumonia*	6.18±0.81 A	7.21±0.51 A	7.98±.31 A
Phosphate buffer saline	0.75±0.25 B	1.25±0.47 B	0.75±0.47 B

Different capital letters means a significant difference between groups (P≤ 0.05).

Table, 5: Diameters of indurations (Skin test) in rabbits immunized with sonicated. *K. pneumonia* and *E. tenella* by 1/2 crude sonicated *K. pneumonia* antigen.

Group	Diameter of erythema (Means±SE mm)		
	Hours		
	24	48	72
E.tenella	4.61±0.83 A	5.23±0.30 A	6.46±0.81 A
K.pneumonia	3.88±0.43 AB	4.98±0.41 A	5.22±0.28 A
E. tenella and *K.pneumonia*	5.80±0.30 AC	5.92±0.68 A	6.90±0.82 A
Phosphate buffer saline	0.50±0.28 D	0.75±0.25 B	0.50±0.28 B

Different capital letters means a significant difference between groups (P≤ 0.05).

Table, 6: Diameters of indurations (Skin test) in rabbits immunized with sonicated *K. pneumonia* and *E. tenella* by 1/4 crude sonicated *K. pneumonia* antigen.

Group	Diameter of erythema (Means±SE mm)		
	Hours		
	24	48	72
E.tenella	2.21±0.41 A	3.84±0.60 A	4.89±0.30 A
K.pneumonia	2.10±0.37 A	2.98±0.80 A	3.40±0.81 A
E. tenella and *K.pneumonia*	3.40±0.61 A	4.70±0.81 A	4.89±0.30 A
Phosphate buffer saline	0.50±0.28 B	0.50±0.28 B	0.00±0.00 B

Different capital letters means a significant difference between groups (P≤ 0.05).

Peripheral blood mononuclear cells (PBMCs) were stimulated for the production of proinflammatory cytokines by live and heat-killed isolates and plasmid DNA; modulation by cellular pathway inhibitors. *K. pneumoniae* carbapenemase (KPC)-producing isolates seem to be highly virulent in a low-TNF-α-release environment, suggesting an immuno -paralysis induction mechanism, which may directly contribute to the immune system stimulation (17). At the same time, it was found that immunization with (k) antigen and continue in elevation after the infection with parasite, indicated the possibility to use the outer membrane protein (O antigen) extracted from this bacteria as immunological modificatory that could stimulate both innate (natural) and acquired immunity against amoebic dysentery infection in white rabbits (18). The mechanisms are of the cellular response of bacterial and parasite (T helper 2 for parasite and T helper 1 for bacteria) and there was a balance between these pathways. The parasite infection favors the Th2 cell development and indirectly suppresses the establishment of bacteria (19). Also, the highest mean of indurations found in the group immunized with sonicated *E. tenella* protein played a major role in host specific immune response, because, its infection produced high levels of $CD8^+$ cytotoxic T cell (CTLs) and $CD4^+$ T helper cell, which is considered the major presenting cells of skin test (6), that supported our results. The immunized group with *K. pneumoniae* and *E. tenella* gave a high reaction and this is followed by *E. tenella* immunized group and finally K. *pneumoniae* immunized group.

Table (7) showed high antibodies titers (720 ± 201.32) in the groups that immunized by *E. tenella* and *K. pneumonia* after 35 days with a significant difference (P≤0.05) compared to the *E. tenella* immunized group, but no significant difference (P≥0.05) was recorded between this group and group that immunized by *K. pneumonia* at the same period; while all immunized groups had a significant difference (P≤0.05) in the antibodies titers when compared with the control group at different periods (21, 28 and 35 days).

Table, 7: Antibodies titers of rabbits immunized by sonicated *Klebsiella pneumonia* and *Eimeria tenella*.

Groups	Days		
	21	28	35
	Means ± SE		
E.tenella	50±10.00 A	100±20.00 A	200±40.00 A
K.pneumonia	90±25.16 AB	180±50.33 AB	360±100.66 AB
E. tenella and *K.pneumonia*	180±50.33 B	360± 100.66 B	720± 201.32 B
Phosphate buffer saline	15± 2.88 C	25± 5.00 C	30± 5.77 C

Different capital letters means a significant difference between groups (P≤ 0.050).

In the immunoglobulins production, such as IgG, IgA and IgM, which play a vital role in the binding of foreign antigens, the presence of these antibody molecules on a microbial or parasitic surface can cause clumping (Agglutination) and IgG and IgM activate the complement system (20) but, *K. pneumoniae* fail to evoke capsular antibodies and resistance to infection (21). Similarity, the sonicated *E. tenella* sporozoite protein produces stimulus for development of immunity and this protein gives a significant decrease in lesion score and this protein proved to be very effective and the titer of antibodies reached to the peak at 40 days post immunization that possesed excellent passive immunity (22).

Through carrying this study, it was concluded that the possibility of using *E. tenella* antigens as an immunopotentiater against bacterial infection.

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تداخل الإستجابة المناعية لجرثومة *Klebsiella pneumonia* وطفيلي *Eimeria tenella*

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الخلاصة

هدفت هذه الدراسة إلى معر فة تداخل الإستجابة المناعية (الخلوية والخلطية) لجرثومة الكليبسيلا الرئوية (*Klebsiella pneumonia*) وطفيلي الايميريا تينيلا (*Eimeria tenella*) باستعمال فحص الحساسية المتأخر في الجلد وفحص التلازن في الأنبوبة وذلك باستعمال ستّة عشر ارنباً محلياً. وزعت توزيعاً عشوائياً إلى اربع مجاميع متساوية. منعت المجموعة الاولى بمستضد أكياس بيض طفيلي الايميريا تينيلا المكسرة وبجرعة 1 مل (1000 ميكروغرام / مل)/ تحت الجلد، والمجموعة الثانية منعت بمستضد الخلية الكاملة لجرثومة الكليبسيلا الرئوية المكسرة وبجرعة 1 مل (1000 ميكروغرام/مل)/ تحت الجلد، والمجموعة الثالثة منعت بجرعة 1مل (500 ميكروغرام/ مل بمستضد طفيلي الايميريا تينيلا + 500 ميكروغرام/ مل بمستضد الخلية الكاملة لجرثومة الكليبسيلا الرئوية المكسرة)/ تحت الجلد، وحقنت المجموعة الرابعة (مجموعة السيطرة) 1مل من المحلول الملحي الفسلجي تحت الجلد. وبعد مرور أربعة عشر يوما من المنع، أعطيت المجاميع الممنعة جرعة التقوية بنفس الجرع المذكرة أعلاه. اظهرت نتائج فحص الجلد المتأخر للمجموعة الثالثة وجود اهمية معنوية ($P\leq 0.05$) في تفاعل الإحمرار والتصلد للجلد مقارنة مع المجموعتين الأولى والثانية وذلك بعد مرور 24 و48 و72 ساعة، فضلاً عن وجود تناغم مع مستوى المعيارالحجمي للضدات والذي ارتفع ليبلغ 720±201.32 مقارنة مع المعياران الحجميان للمجموعة الأولى والذي بلغ 200±40.00 والمجموعة الثانية 360±100.66 بعد مرور 35 يوما من الميع. نستنتج من هذه الدراسة والتي تعد الاولى، فعل التداخل التآزري بين مستضدي جرثومة الكليبسيلا وطفيلي الايميريا تينيلا في الاستجابة المناعية (الخلوية والخلطية).

الكلمات المفتاحية: الكليبسيلا الرئوية، الآيميريا تينيلا، اختبار الجلد، التداخل التفاعلي.