# The immunological responses of sonicated *Pasteurella multocida* and *E. tenella* antigens in rabbits

**Dh. Y. Mahmood, I. A. A. Al-Samarraae and H. M. A. Al-Rubaie**
**College of Veterinary Medicine\ University of Baghdad**

## Abstract

This study was aimed to estimate the immune responses of *Pasteurella multocida* and *Eimeria tenella* antigens in rabbits. Twenty albino rabbits were divided randomly into four groups; the first group was immunized with sonicated *P. multocida* antigen (500µg), the second group immunized with sonicated *E. tenella* (500µg), third group immunized with both antigens (500 µg) and the fourth group as control that injected with PBS (pH 7.2). The cellular immune response against *P. multocida* was evaluated by using the delayed type hypersensitivity (DTH)-Skin test at 21 days post immunization. The results were showed an elevation of the mean diameter of erythema at 24hr of immunized groups and the third group was showed the highest diameter (7.32±0.54 mm) compared with the first and the second groups (5.96± 0.70 mm and 6.52± 0.41 mm) respectively. Also, there was an increase in the diameter of induration at 72 hrs and the highest diameter was recorded at the third group (6.90±0.41 mm) compared with first and second groups (2.90±0.46mm and 4.35±0.35mm) respectively with a significant differences ($P<0.05$). The humoral immune response was estimated by using ELISA and Tube agglutination test. The higher antibodies titer was recorded at 49$^{th}$ post immunization, the highest value was found in the third group (104.31± 6.32 ng) when compared with first and second group (68.86± 9.69 ng and 80.33 ±15.95 ng) respectively with a significant differences ($P<0.05$). The peak of antibody titers of tube agglutination test was recorded at the 49$^{th}$ day and the highest antibody titers was found in third group (512±70**)** when compared with first (416±86) and in the second group (352±70); with a significant difference ($P<0.05$). We concluded that there was an interaction between both antigens to enhance the humoral and cellular immune response.

**Key words:** Sonicated, *Pasteurella multocida* antigen, *E.tenella* oocysts, skin test, tube agglutination test.

# الاستجابة المناعية لمستضدي المكسرة في الأرانب *Pasteurella multocida* و *Eimeria tenella*

**ضحى يحيى محمود، إكرام عباس عبود السامرائي وحيدر محمد علي الربيعي**
**كلية الطب البيطري/ جامعة بغداد**

## الخلاصة

هدف الدراسة تقييم الاستجابة المناعية الخلطية والخلوية في الأرانب الممنعة بمستضدي *Pasteurella multocida* وأكياس بيض طفيلي *Eimeria tenella* المكسرة من خلال استعمال عشرين أرنب ابيض اللون، قسمت عشوائيا إلى أربعة مجاميع. منعت المجموعة الأولى بمستضد *Pasteurella multocida* المكسرة (500 مايكروغرام)، منعت المجموعة الثانية بمستضد أكياس بيض طفيلي *Eimeria tenella* المكسرة (500 مايكروغرام)، منعت المجموعة الثالثة بكلا المستضدين (500 مايكروغرام) وحقنت المجموعة الرابعة (مجموعة السيطرة) بالمحلول الملحي الوظيفي (الأس الهيدروجيني 7.2). قيمت المناعة الخلوية بفحص فرط الحساسية المتأخر. فأظهرت النتائج أن اعلى معدل للاحمرار كان في المجموعة الثالثة (7.32 ±0.54 ملم) مقارنة

بـالمجموعتين الأولـى (5.96 ± 0.70 ملـم) والثانيـة (6.52 ± 0.41 ملـم). أمـا معـدل التـثخن، فقـد أعطـت المجموعـة الثالثـة أيضـا اعلـى معـدل (6.90 ± 0.41 ملـم) بالمقارنـة مـع المجموعـة الأولـى والثانيـة علـى التـوالي (2.90 ± 0.4، 4.35 ± 0.35 ملم) وبفرق معنوي (P<0.05). أما المناعة الخلطية فقد فحصت بأجراء فحص مقايسـة الممدص المنـاعي المرتبط بـالإنزيم وفحص التـلازن بالأنبوبـة، فـأظهرت النتـائج أن اعلـى معـدل لتركيـز للضدات كان في اليوم 49، وان المجموعة الثالثة أعطت اعلى معدل لتركيز الضدات بفحص الأول (104.31 ± 6. 32 نانوكرام) بالمقارنة مع المجموعة الأولى والثانية (80.33 ± 15.95، 68.86 ± 9.69 نانوكرام) على التوالي، أما بفحص التلازن بالأنبوبة فكان اعلى تركيز للأضداد في المجموعة الثالثة (512±70) بالمقارنة مع المجاميع الأولى والثانية (416 ± 86، 352 ± 70) على التوالي وبفرق معنوي (P<0.05). نستنتج من ذلك بأن هنالك تداخل مناعي بين كلا المستضدين مما يؤدي إلى تحفيز الاستجابة المناعة الخلطية والخلوية.

## Introduction

Pasteurellosis is a serious contagious disease that rapidly speared between members of animals resulting in large scale outbreaks. It is the most wide spread disease over the world, even the country with low humidity and low temperature and cattle and buffaloes is the most susceptible animals then the poultry and rabbits (1). It's mortality rates between 50-60%, which cause a severe economic losses in animals husbandry and industry, these losses reach a one billion U.S dollars per year (2). *Pasteurella multocida* is the causative agent of disease, it has a five serogroups (A, B, D, E and F) and 16 serotypes; these groups were detected among different livestock population (3). Serotype B: 2 and E: 2 are the most common serotypes, that were associated with disease in Asia and Africa (4) and the B: 2 serotype associated with hemorrhagic septicemia which is widely distributed in Asia (5). The antigenic structures of *Pasteurella multocida* are capsular and somatic antigens (6) and the nature of immune responses are relatively contributed to humoral and cellular immunity (7). *Eimeria tenella* is a highly immunogenic and primary infection can be stimulate a protective immunity to the subsequent challenge (8). The immunization simulate the different T helper cells population and give a high levels of various types of cytokines (interleukins) (9); also, give a high titers of multiple immunoglobulin including IgG, IgM and mucosal IgA and give an immunity against the infection to offspring (10). The treatment failure of *Pasteurella multocida* due to the resistance to antibiotics (80.5% of them), as well as, their toxic effect for human consumer (11), and the highly cost of management; Vaccination considered the successful tool for prevention and controlling the infection and improving the health with prevent the transmission of disease from domesticated and wild animals to human (12). For all these purposes; This study was conducted to evaluate the immune responses of the sonicated *E.tenella* oocysts antigen as an adjuvant to potentiate the immunized rabbits by *Pasteurella multocida* antigen.

## Materials and Methods

- Microorganism's isolates: *Pasteurella multocida* isolate supplied by Alkindi Company for Veterinary Drugs and Vaccines Production, Baghdad, Iraq, and the biochemical tests were done to confirm their diagnosis according to (13).

A. *P.multocida* whole cell antigen (*P.m.*W.C.Ag): It was prepared according to (14) and then sonicated according to (15).

B. *Eiemeria tenella* oocysts: *Eiemeria tenella* oocysts were isolated from a seca of infected broiler chicken, that were adequate from the field in Baghdad city. These oocysts had been collected according to (16) and then sonicated (15).

C. Protein concentration: Protein concentration was estimate in both antigens by using the Biuret method (16).

- Laboratory animals: Twenty healthy albino rabbits were randomly divided into four groups; the first group immunized with 500 µg/ml of sonicated *Pasteurella multocida* antigen, the second group immunized with 500 µg/ml of sonicated *Eimeria tenella* oocysts antigen, the third group immunized with 500 µg/ml of both sonicated antigens (250 µg/ml of *Pasteurella multocida*, 250 µg/ml of sonicated *Eimeria tenella* oocysts), subcutaneously and the fourth group (Control group) was injected subcutaneously with 1 ml of phosphate buffer saline (pH 7.2). All immunized animals were given a booster dose after 14 days of immunization. At day 21$^{th}$ post immunization the skin test had been done according to (17). The blood samples were collected two weeks intervals for three times, for antibodies titers determination by using tube agglutination test (18) and ELISA according to Immunology Consultants Laboratory, Inc. USA.
- Statistical Analysis: The results were analyzed by using the SPSS for different groups at a level 5% and 1% (19).

## Results

- **Delayed type hypersensitivity test (DTH-Skin Test):** The mean diameter of erythema of the skin (mm) after 24 hrs showed that the highest diameter was recorded in the third group that immunized with both sonicated *P. multocida* and *E. tenella* antigens (7.32±0.54) when compared with second and first group (6.52±0.41 mm and 5.96± 0.70 mm) respectively. Also, there was an increase in the diameter of induration at 72 hrs and the highest diameter was recorded at the third group (6.90±0.41) compared with first and second groups (2.90±0.46 mm and 4.35±0.35 mm) with a significant differences ($P<0.05$). With a significant difference (Table 1, 2).
- **Antibodies titers:** The results of ELISA were showed an increase in mean titer of the antibodies in at 49$^{th}$ especially with the third group that immunized with both antigens (104.31±6.32) when compared with the first and the second groups (68.86±9.69 and 80.33±15.95) respectively. While in tube agglutination test the titers of IgG showed high elevation at 35$^{th}$; showed the highest titer with the third group that immunized with both sonicated *P. multocida* and *E.tenella* oocysts antigens (320 ± 78). When compared with first and second group (288 ±83.6 and 256±94) respectively. At 49$^{th}$ the mean of titer reach to the peak and the third group showed the highest mean (512±70) and give significant differences ($P<0.05$) with first group (256±94). At 63$^{th}$ the mean was decreased and the second group showed elevation when compared with first group and the third group remains the highest mean (Table 3, 4)**.**

**Table (1) Erythema (mm) of Delayed Type Hypersensitivity (DTH)-Skin test of immunized rabbits with different antigens**

| Time / Groups | 24 hr. | | | | 48 hr. | | | | 72 hr. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean ±SE (MM) | | | | | | | | | | | |
| | Crude | 1:2 | 1:4 | P.B.S | Crude | 1:2 | 1:4 | P.B.S | Crude | 1:2 | 1:4 | P.B.S |
| G1 | 5.96±0.70 *a A | 4.90±0.38 ab A | 3.80±0.46 B A | 0.1 | 4.70±0.48 a A | 3.24±0.43 a** A | 1.80±0.30 b* A | 0.1 | 3.5±0.43 a* A | 2.88±0.35 ab A | 1.90±0.35 B | 0.1 |
| G2 | 6.52±0.41 *a A | 5.42±0.38 ab A | 4.40±0.38 b A | 0.1 | 5.22±0.40 a A | 3.74±0.36 b* A | 2.42±0.40 b** A | 0.1 | 4.52±0.47 a A | 3.98±0.45 a A | 3.20±0.42 a A | 0.1 |
| G3 | 7.32±0.54 a A | 5.90±0.49 ab A | 4.90±0.59 B A | 0.1 | 5.86±0.46 a A | 3.90±0.45 b* A | 2.84±0.36 b** A | 0.1 | 4.96±0.46 a A | 3.70±0.48 a b A | 2.80±0.22 b ** A | 0.1 |

- **P<0.05 *P<0.01
- Capital letters denote a significant difference between groups.
- Small letters denote a significant difference with in group.

**Table (2) Indurations (mm) of Delayed Type Hypersensitivity (DTH)-Skin test of immunized rabbits with different antigens**

| Days / Groups | 24 hr. | | | | 48 hr. | | | | 72 hr. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean ± SE(mm ) | | | | | | | | | | | |
| | Crude | 1:2 | 1:4 | P.B.S | Crude | 1:2 | 1:4 | P.B.S | Crude | 1:2 | 1:4 | P.B.S |
| G1 | 3.10±0.40 a* A* | 2.30±0.39 ab A* | 1.60±0.16 b A* | 0.1 | 4.32±0.36 a** A | 3.43±0.44 ab A | 2.28±0.31 A* b | 0.1 | 5.20±0.42 a* A* | 3.34±0.47 b A | 2.90±0.46 b A | 0.1 |
| G2 | 4.66±0.54 a* AB | 3.62±0.41 ab AB | 2.70±0.26 B b | 0.1 | 5.16±0.62 A a | 4.90±0.45 A a | 3.86±0.34 B a | 0.1 | 6.66±0.35 a B | 5.98±0.37 a B** | 4.35±0.35 b** AB | 0.1 |
| G3 | 5.04±0.48 a B | 4.10±0.40 B a | 3.90±0.35 a A | 0.1 | 5.70±0.48 **a A | 4.50±0.66 A ab | 3 ±0.42 b AB | 0.1 | 6.90±0.41 a* B | 5.84±0.54 ab AB | 4.76±0.67 B b | 0.1 |

- **P<0.05 *P<0.01
- Capital letters denote a significant difference between groups.
- Small letters denote a significant difference with in group.

**Table (3) The antibody titer measured by ELISA of immunized groups with different antigens**

| Mean ± S.E (ng) | | | |
|---|---|---|---|
| Days / Group | 35th | 49th | 63th |
| G1 | 67.03±8.96 a A | 68.86±9.69 a A | 66.01±10.57 a A |
| G2 | 72.57±7.27 a A | 80.33±15.95 a B | 57.86±9.12 a A |
| G3 | 96.52±5.70 A B | 104.31±6.32 a B | 91.06±2.53 a B |
| G4 | 9.23±0.44 a C | 9.59±0.58 a C | 9.13±0.67 a C |

- ****P<0.05 *P<0.01**
- **Capital letters denote a significant difference between group.**
- **Small letters denote a significant difference with in group.**

**Table (4) The antibody titer measured by Tube agglutination of immunized groups with different antigens**

| (Mean ± S.E) | | | |
|---|---|---|---|
| Days / Groups | 35th | 49th | 63th |
| G1 | 288±83.6 a A | 416±86 a A | 208±43 a A |
| G2 | 256±94 a A | 352±70 a A | 272±89 a A |
| G3 | 320±78 a A | 512±70 a A | 240±45 * a A |

- ****P<0.05 *P<0.01**
- **Capital letters denote a significant differences between groups.**
- **Small letters denote a significant differences within group.**

## Discussion

Skin test occurred due to the role of memory cell that modulate Th1 to secrete Interferon-γ (INF-γ), potent mediator that stimulates the migration of macrophage to the site of reacted area of skin (20). while the macrophage secrete Interleukin1 (IL1) that enhance proliferation and differentiation of other T cells into T helper-1(Th1) cells which secrete Interleukin-2 (IL2) a chemotactic factor that cause attraction of macrophages around area of activated T cells (21). The antigen presenting cells was firstly taking up the foreign protein and broke it into many peptides 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 of hours to develop the action on injected site (22). The lipopolysaccharide (LPS) and the porins of *Pasteurella multocida* cause regulation of mRNA of expression levels of proinflamatory cytokine (TNF-α, IL1β and IL8) these influx inflammatory cells (polymorphonuclear and mononuclear cells) and enhance their infiltration (23). Also; *E. tenella* antigen protein that play major in host immunity and considered the target of in specific immune response because of the infection of *E. tenella* produce high level CD8+ cytotoxic T cells (CTLs) and CD4+ T helper cell that considered the major presenting cells of skin test (24).

The antibodies titer markedly elevated after 35th, in all immunized groups compared with control group, with a peak at 49th days especially in the third group that immunized with sonicated *P.multocida* and *E.tenella* antigens which had the highest antibodies titer compared with the first group immunized with sonicated *P.multocida* antigen and in the second group which immunized with sonicated *E.tenella* antigen; that mentioned in many of previous studies proved that the outer membrane proteins of *P.multocida* could posses protective and potent strong potential role in immunogenicity against infection and could be used as a booster dose after primary vaccination by enhancing the duration of protective immunity. This protein has been evaluated as a vaccine through different routes of immunization and specially induces the mucosal immunity (25). The sonicated *E.tenella* sporozoite protein produce strongest stimulus for development the immunity and this protein have been demonstrate that give a significant decrease in lesion score, oocysts output and provide protection about 99.2-99.5% and this protein was very effective and the titer of antibodies reached to the peak at 40 days post immunization and posses excellent passive immunity (26). *P. multocida* antigen with FMD virus in rabbits, which compared with monovalent FMD & H.S alone, this study showed that the bivalent FMD+HS vaccine peak level was detected on 48th days post-priming. It is concluded that combine bivalent vaccine produced better antibody titer that persisted for longer time (27).

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