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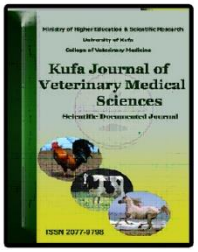

The synergestic effect of probiotic and Levamisole on antibody titre of Newcastle disease in presence of E.coli as stress

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Abstract

This study was conducted to investigate the effects of probiotic (poultry star®), and immunmodulator (levamisole) in feed additive and in drinking water as synergistic or each one alone on immune response post routine vaccination and challenge with Newcastle disease virus and *E.coli*, also on means lesion score in (air sac, heart and liver). one hundred Ross broiler chicks were used from 1 day to 35 day old, divided randomly to five groups (20 birds/group), diet and drinking water offered ad libitium, experimental groups treated as follows: Group one: received poultry star® as feed additive in dose (2×10^{11} CFU/kg) from one day till the end of the experiment. Group two: received levamisole (10µg/kg B.w.) in drinking water from one day till the end of the experiment. Group three: received levamisole (10µg/kg B.W.) in drinking water plus poultry star in feed additive in dose (2×10^{11} CFU) from one day till the end of the experiment. Group four: control positive positive (vaccinated not treated). Group five: Control negative (not vaccinated not treated). All groups except the control (negative) vaccinated with ND vaccine (*La Sota* strain) orally in drinking water at (5 and 15) days and vaccinated with IBD vaccine (intermediate strain) at 12 days. The challenge was carried out through intranasal inoculation with virulent ND virus ($100LD_{50}$, 1×10^{6}) at day 20, exposed to intratracheal inoculation with *E.coli* (3×10^{7} CFU/ml) at day 22. The obtained results referred to the synergistic effect of probiotic and levamisole in group three that enhanced the immunity against Newcastle virus, also less means level of air sac lesion, pericarditis and perihepatitis score as compared with single effect in the groups (first and second) and also compared with control negative and positive groups. NDV and *E.coli* Challenge caused high morbidity and no mortality in all vaccinated group compared with control negative. Therefore, all the additive materials (probiotic, levamisole) protected the chicks from bacterial infection *E.coli*. But did not protect the chicks from infection with virulent NDV in spite of that reducing the severity of infection by reduced the viral replication.

Key word: Newcastle disease, Probiotic, levamisole, Broilers, ELISA, lesion scores.

الخلاصـــة:

قد اجريت هذه الدراسة لمعرفة تاثير المعزز الحيوي المتعددة السلالات (poultry star®) والليفاميزول كاضافات علفية في العلف وفي مياه الشرب, بشكل تازري او كل واحد على حده على الاستجابة المناعية قبل وبعد التحدي بفايروس الضاري لمرض نيوكاسل والاشريشا القولونية، وايضا على معدل علامات الافات في

(كيس الهواء والقلب والكبد). تم استخدام مئة فرخة اللاحم من سلالة روس 308 من عمر (1-35) يوم تقسيمها عشوائيا لخمسة مجاميع (20 طائر / مجموعة)، المجاميع التجريبية تم معالجتها كما يلي: المجموعة الاولى: تم اعطائها (poultry star®) كاضافات علفية في العليقة بجرعة (2×10^{11} cfu/kg) من عمر يوم واحد حتى نهاية التجربة. المجموعة الثانية: تم اعطائها الليفاميزول بجرعة (10µg / كغم من وزن الجسم) في مياه الشرب منذ اليوم الاول حتى نهاية التجربة. المجموعة الثالثة: تلقي الليفاميزول (10µg / كجم من وزن الجسم) في ماء الشرب بالاضافة الى (poultry star®) كاضافات علفية في العليقة بجرعة (2×11^{11} cfu/kg) من عمر يوم واحد حتى نهاية التجربة. المجموعة الرابعة: المجموعة سيطرة موجبة. المجموعة الخامسة: مجموعة سيطرة سالبة. جميع المجاميع ما عدا مجموعة السيطرة السالبة تم تلقيحها بلقاح النيوكاسل (سلالة لا سوتا) في ماء الشرب بعمر (5 و 15) يوم، ولقحت بلقاح التهاب جراب فابريشيا (سلالة المتوسط الضراوة) بعمر 12 يوم. في حين ان التحدي بفايروس النيوكاسل الضاري (1×10^{6} $100LD_{50}$) عن طريق الحقن داخل الانف بعمر 20 يوم، وتم تعريضها الى الاشريشا القولونية عن طريق الحقن داخل القصبة الهوائية (3×10^{7} خلية / مل). النتائج التي تم الحصول عليها تشير الى ان التاثير التازري للمعزز الحيوي والليفاميزول في المجموعة الثالثة اعطى اعلى استجابة مناعية ضد فايروس النيوكاسل، وكذلك اعطت اقل معدل علامات للافات في (كيس الهواء والقلب والكبد) المتمثلة ب(airsacculitis وpericarditis وperihepatitis) بالمقارنة مع التاثير الاحادي في المجموعة الاولى والثانية وبالمقارنة مع مجموعات السيطرة. نتيجة التحدي بفايروس النيوكاسل الضاري والاشريشا القولونية سبب ارتفاع في نسبة الاصابة بدون هلاكات في كل المجاميع الملقحة ولكن ارتفاع في نسبة الاصابة والهلاكات في السيطرة السالبة, لذلك جميع المواد المضافة (المعزز الحيوي والليفاميزول) تحمي الافراخ من الاصابات البكتيريا بالاخص الاشريشيا القولونية. ولكن لا تحمي الافراخ من خطر الاصابة بافايروس النيوكاسل الضاري لكن تعمل على خفض شدة الاصابة من خلال التقليل من تكاثر الفايروس.

Introduction

Newcastle disease (ND) is one of the most important poultry disease causing severe economic losses in poultry industry, etiological agent is Newcastle disease virus member of Genus Avulavirus, family Paramyxoviridae (1). Most of countries where poultry is raised commercially either broiler or layer or breeders and where is the disease is endemic rely on different vaccination programs on order to control the disease (2). In spite of use of many vaccination programs include both commercial live and inactivated oil adjuvant vaccines (3), there is continued outbreak of velogenic Newcastle disease in reared poultry farms which give raise for need of more effective vaccination program in protecting birds against challenge (4). Generally, E. coli affects poultry of all ages, although young birds are more sensitive. The infection is considered to be one of the leading causes of economic loss in the poultry industry. More frequently, E. coli disease occurs as a consequence of the adverse influence of factors such as ammonia, moisture, dust, hormones or infectious agents such as viruses and mycoplasmas (5). Sometimes E. coli is the primary cause of disease particularly in young and adults birds (6). These arguments put pressure on the future of feeding low-level antibiotics to animal Probiotic which are live microbial feed supplements that beneficially affect the host animal by altering its intestinal microbial balance (7), The same finding with Guo et al., (8) confirm that some isolates of probiotics have inhibitory activity against strains of E.coli K88 and K99 that was explained by Zohair (9) who found that probiotics in broiler chickens ration has capability to reduce colonization of E.coli in intestine together with reducing both mortalities, severity of postmortem and histopathological lesions. Talebi et al., (10) stated that not only the use of probiotics significantly enhanced broiler performance by improving body weight and decreasing feed conversion ratio but also improve the

antibody responses to Newcastle disease virus and infectious bursal disease vaccination but the antibody titers of the probiotic treated group were not significantly different from those not received probiotics. Chawak et al., (11) levamisole (LMS) may act as a multifunctional modulator after immunization to mediate the cell-mediated response of T cells, and at the same time promote activated B cells to produce antibody; this is another possible method of LMS to stimulate immune system. levamisole can enhance lymphocyte proliferation both in mice and chickens (12) indicating its ability to induce cellular immunity, which was confirmed by its ability to induce a high level of IFN-gamma (IFN-γ). Furthermore, LMS do not only improve the humeral response but also induces a cell-mediated response to killed NDV vaccine, which in turn produces sustainable immune responses (12).The aim of the present study is the evaluation the positive effect of probiotics and Levamisole on broiler immunity in the period of infection with (ND) and E. coli.

Materials and methods

One hundred Broiler (Breed: Rose 308, Origin: Belgium) were brought in good condition from AL-Anwar Hatchery-Dialya- Kanan. The chicks were divided randomly into 5 groups, each group contained 20 birds in poultry field distance of 30 x 8 m divided into five groups by plastic barriers and the ground mattress with wood sheavers litter and supplemented all management requirements as poultry hygiene standardization. Blood samples were collected from jugular vein randomly from 10 chicks in 1 day old for measuring of maternal immunity against Newcastle disease virus (NDV) using ELISA test (Indirect method). All chicks were vaccinated except the control negative with NDV vaccine La Sota strain (Intervet - Holland) by manual oral drench in 5 days old regarding to the ELISA Abs titer against NDV (maternal immunity), the second NDV vaccine applied at 15 days old by the same procedure and in both vaccinations the vaccine was dissolved in saline. The single dose after vaccine dissolving is 1 ml / bird contained $10^{6.5}$ EID50. Also all chicks were vaccinated except the control negative with IBDV Gumbo intermediate D78 strain (Synoviae - USA) in 10 days old by the same manner used in NDV vaccination. experimental groups treated as follows: Group one: received poultry star® as feed additive in dose (2×10^{11} CFU/kg) from one day till the end of the experiment. Group two: received levamisole (10µg/kg B.w.) in drinking water from one day till the end of the experiment. Group three: received levamisole (10µg/kg B.W.) in drinking water plus poultry star® in feed additive in dose (2×10^{11} CFU) from one day till the end of the experiment. Group four control positive. Group five: Control negative. challenged by intranasal inoculation with virulent ND virus (100LD50, 1×10^{6}) have (the mean death time 48 hours and ICPI 1.67) at day 20, exposed to intratracheal inoculation with *E. coli* (3×10^{7} CFU/ml) The *E-coli* strain was isolated from the clinically affected broiler farms with colibacillosis in Baghdad Province. Then the isolate was confirmed by culturing on selective culture media and the biochemical tests were carried out using the API 20 system to be sure that it was Avian pathogenic *Escherichia coli* and their pathogenicity was determined by Acraflavine test.

The samples of blood serum were taken to measure the antibody titre against Newcastle disease by ELISA test accordance to (13). the mean lesion score application was carried out according to (14) and (15).

Results and Discussion

The results of the current study reflected the presence of significant differences at level (P<0.05) among all groups in Ab titre against ND at (15, 20, 25 and 35) days old chicks.

At 15 days old chicks, the highest mean antibody level titre among the vaccinated groups was gave by group three which was (3411.6) followed by medium mean antibody level titre in (second and first) groups, which were (2382.5 and 1534) respectively, Whereas, the lowest level antibody titre gave by control positive group which was (999.6) as compared with the control negative group which did not record any immune response table (1).

At 20 days old chicks, the highest antibody level titre was gave by group three which were (4605.8). followed by (second and first) groups, which were (3460.4 and 2639.2) respectively, as compared with the control positive which was recorded (1961.4) while control negative which did not recorded any immune response table (1).

In the other hand, the decrease Ab titers in all immunized groups at day 25 as compared with antibody level titre at day 20. The lowest antibody level titre was gave by groups control negative and positive were (0 and 769) respectively, compared with the (third, second and first) groups which recorded (2128.7, 1778.4 and 1022.4) respectively table (1).

Whereas the increase Ab titers in all immunized groups at day 35 as compared with antibody level titre at days (15, 20 and 25). The highest antibody level titre was given by third group which was (6308.8), followed by (third, second and first) groups, which were (5101, 3316.7 and 2731.1) respectively as compared with the control positive which was (2103.5). But the control negative group birds were dead table (1).

Table 1. Results of antibody titer against Newcastle disease in different days by ELISA test

Groups	15 days	20 days*	25 days	35 days
1	1534±26.5 C c	2639.2±109.2 C b	1022.4±22.2 C d	3316.7±157.9 C a
2	2382.5±49.8 B c	3460.4±102.5 B b	1778.4±37.7 B d	5101±116.3 B a
3	3411.6±45.5 A c	4605.8±131.9 A b	2128.7±47.6 A d	6308.8±116.6 A a
Cp	999.6±27.57 D c	1961.4±53.7 D b	769±37.3 D d	2103.5±45.6 D a
Cn	0±0 E	0±0 E	0±0 E	All dead

*Number of samples=10. * challenged with virulent ND local isolate

-The different capital letters refer to significant differences between different columns (P<0.05) -The different small letters refer to significant differences between different raws (P<0.05)

The Newcastle disease virus constitutes a serious problem in most countries of the world. It is responsible for high mortalities among poultry population. Though several types of vaccines were produced against the disease as Hitchner B_1, Lasota, F, K and inactivated vaccines (2). Yet still disease is widely spread and outbreaks appear even in farms received a program of several vaccination and the virus remains an ongoing threat to commercial flocks (3).

The result of 10 serum samples out of 100 three-day old chicks (before division into groups) for assessment maternal immunity against NDV revealed high level, the mean value was (4420.9) for the first experiment. These findings are in agreement with Hamal *et al*., (16) who reported that the cumulative effect of Ab production resulted from several times of vaccination reach to a high titres, thus the eggs will receive a large amount of Mab from their own breeders, and this will be representing in the hatched chicks.

High level of maternal antibody in day-old-chicks was also reported by (17). The rate of declination of maternally derived antibody was about half by every 5 days. This finding is in agreement with the findings of Allan *et al.*, (18) who estimated that each two-fold decay in maternally derived HI antibody titre takes about 4-5 days. Generally all groups showed increase in antibody titre except the control negative group which reflected undetectable antibody titres by ELISA test. Many investigators like Giambrone (19); Wyeth and Chettle (20) interpreted the role of live attenthuated vaccine (La Sota) at 10 days which leads to multiplication of the virus (without interference with Mab that gradually decreased by time), thus stimulation of immune organs in response to produce more Abs. Another reason could be put forward to comprehend the immune status of our immunized chicks post-vaccination that the La Sota vaccine was given orally by drinking water, hence this route will stimulate the cell mediated immune response that requires longer time for replication of the virus in vivo, then stimulation of immune system will occur to furnish more Abs in the circulation (21).

Ahmad, (22) explained the delayed first immunization against ND in the first few days (especially in the seventh and eighth days) of chicks will give better immune response, however, it is thought that vaccination at 10 days give a better result than if it was done earlier. While the increase in Ab titres in all groups in different levels at (15 and 20) days old especially third group as compared with control positive group due to the synergistic role between probiotic (poultry star®) and immune regulator (levamisole) in strengthening the immune birds against Newcastle disease, these finding agreemt with (23) who explained that a *lactobacillus acidophilus* bacteria promote improvement in the immune system by three different ways: enhance macrophage activity and ability to phagocyte microorganism and activate B lymphocytes and plasma cells leading to increase production of antibodies usually IgG, on other hand, probiotic in diet increases white blood cells count (24).

Pathogen-associated molecular patterns (PAMPs) derived from probiotic bacteria are recognized by pattern recognition receptors, such as Toll-like receptors (TLRs) on dendritic cells (DC) in the epithelium or lamina propria. The recognition of commensal bacteria or their structural components

by (TLRs) present on surfaces of (DC) could lead to the activation and maturation of these cells (23). Differential activation of (DC) (professional antigen presenting cells) by commensal bacteria promotes the establishment of T-helper 1 (Th1) and Th 2 responses and the secretion of cytokines, such as interleukin 4 (IL-4), IL-10, and transforming growth factor β, that are important for antibody production and isotype switching (25).

Chawak et al. (11) Levamisole (LMS) may act as a multifunctional modulator after immunization to mediate the cell-mediated response of T cells, and at the same time promote activated B cells to produce antibody; this is another possible method of LMS to stimulate immune system. Higher functional antibody level (HI) in chickens which has also been seen in our study, completely proves mentioned notions. Levamisole can enhance lymphocyte proliferation both in mice and chickens (12) indicating its ability to induce cellular immunity, which was confirmed by its ability to induce a high level of IFN-gamma (IFN-γ). Furthermore, LMS do not only improve the humeral response but also induces a cell-mediated response to killed NDV vaccine, which in turn produces sustainable immune responses (12).

Cytokines have critical roles in the development and maintenthance of immune responses. IFN-γ, a signature Th1 cytokine, mediates the killing of organisms and is responsible for protection against a variety of intra-cellular infections. Interleukin-4 (IL-4), a Th2 cytokine, can promote B cell differentiation and enhance the production of antibodies by sensitized B cells (26). The results of the second group (levamisole alone) showed good results and agreed with (27) who interpretated that levamisole enhances the innate immune response as it does with the acquired response (acting as an adjuvant). It is documented that cells treated with LMS enhanced their cyclic guanosine monophosphate (cGMP) levels, which also increased microtubular assembly and cell mobility. Also Yin *et al.*, (12) said that there another pathway of higher antibody levels in levamisole treated groups because LMS stimulate the T cells to induce cytokines, all these activities could be possible reasons of significant results in our study.

This study demonstrated that exposure of young broiler chickens with previous intranasal inoculation with NDV, to *E. coli* intratracheal induced have predominantly respiratory lesions. low mortality, and a low percentage of septicemic lesions. Inclusion of NDV as a predisposing agent prior to exposure of chickens to E. *coli* intratracheal increased the frequency and severity of lesions that were produced table (2). Newcastle disease virus causes an acute respiratory disease that in combination with *E. coli* produce airsacculitis in naturally exposed broiler chickens (28). Virulent strains of NDV are able to damage ciliated epithelium of the trachea causing loss of cilia, and rounding and sloughing of epithelial cells predisposing the trachea to opportunistic pathogens such as E. *coli* (29).

The results of current study explained presence of a significant decrease at level ($P<0.05$) among all groups in mean thorasic air sac lesion scores at day 35 chicken, the lowest mean was recorded in third group which was (0.5) followed by (second, first and control positive) which were (0.75, 1 and 2) respectively, as compared with control negative which recorded (2.75)

table (2). Also the mean heart lesion score was recoded significant decrease at level (P<0.05) among all groups at day 35 the lowest mean was recorded in third group which was (0.25) followed by (second, first and control positive) which were (0.5, 0.75 and 1.5) respectively, as compared with control negative which recorded (2.75) table (2). As well as the results of mean liver lesion score was recoded a significant decrease at level (P<0.05) among all groups at day 35 the third groups did not recorded any score (0) followed by (second, first and control positive) which were (0.33, 0.66 and 2.33) respectively, as compared with control negative was recorded (3.66) table (2).

Table 2. lesion scores for groups of chickens inoculated intranasally at 20 days of age with NDV and intratracheally of an *Eschericlria coli* at 22 days of age

Groups	thoracic air sac	Heart	Liver
1	1±0.57 AB	0.75±0.47 AB	0.66±0.66 AB
2	0.75±0.47 AB	0.5±0.5 AB	0.33±0.33 AB
3	0.25±0.5 B	0.25±0.25 B	0±0 B
Cp	2±0.7 AB	1.5±0.94 AB	2.33±1.2 AB
Cn	2.75±1.37 A	2.75±1.25 A	3.66±2.51 A

*Number of samples=5.

The different capital letters refer to significant differences between different columns (P<0.05)

Reducing these effects of *E.coli* through the use of probiotics can affect air sac lesion development. This activity may be due to production of antimicrobial compounds which are produced in culture broth. Jin *et al*., (30) have reported that the antagonistic activity of lactic acid bacteria is due to the production of antibacterial substances. All these substances are able to prevent the growth of enteric pathogens. Lactic acid bacteria are also able to produce lactic acid bacteriocines and reduce pH which inhibits pathogenic bacteria in the intestinal tract (31). These bacteria also produce peptides which have inhibitory properties against other pathogens (30). Many investigations have shown antagonistic activities of lactic acid bacteria. Fermented milk has been used for the treatment of *H. pylori* infections because *Lactobacilli* are present in fermented milk having antibacterial activity against *H. pylori* (23).

Therefore, the synergistic effect of probiotic and levamisole had a major role in reducing the means level of thorasac air sacculitis, pericarditis and perihepatitis in bird after challenge with virulent Newcastle virus and *E.coli*, Levamisole modulates the immune system Booth and McDonald, (32), increase the leucocytes number and serum lysozyme levels (33). The immune stimulation of levamisole at small doses may be attributed to the activation of the non-specific immune response particularly macrophages this activation could enhance the antigen trapping and processing. This agreed with the findings expressed by Zhang *et al.*, (34) who noticed that levamisole enhanced B-lymphocyte

differentiation, and supported by Sun et al. (2003), they suggested that levamisole may modulate serum interleukin-6 (IL-6) level which is according to Sheehan, (35), that secreted by TH_2 (T-helper-2) and can promote B-cell activation, proliferation and differentiation into antibody producing plasma cells. These results were confirmed by those obtained by Drews (1990) suggested that levamisole can enhance production and secretion of IL-2 (Interleukin-2) and interferon. According to Sheehan (1997), IL-2 and interferon-gamma are secreted by TH1 (T-helper-1) and function to promote activation of Tc (T-cytotoxic) cells, NK (Natural Killer) cells and macrophage, and consequently phagocytic activity.

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