Evaluation of lipopolysaccharide and formaldehyde inactive *Escherichia coli* vaccines in broilers

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Abstract

This study was conducted to evaluate the lipopolysaccharide (LPS) and formaldehyde inactive *E. coli* vaccine in different doses by determination the immune response of *E. coli* and estimate the pathological changes post challenge with *Escherichia coli*. Three hundred and fifty broiler chicks were used from one day to forty five day old, divided randomly into 7 groups, experimental groups treated by receiving LPS vaccine of *E. coli* in different doses. And other groups received killed vaccine of *E. coli* through subcutaneous injection in different doses. G3 that received LPS vaccine at dose (0.5 ml from 3×10^7 CFU/ml) given significant increase at level ($P<0.05$) in protection after challenge with *E. coli* compared with other groups. The LPS vaccine from local isolate gave better (cellular and humoral) immunity than the formaldehyde inactivated vaccine in vaccinated chicks after challenge with APEC.

Key word: *E. coli* vaccine, Newcastle disease, lesion score, broilers, ELISA
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تقييم لقاح متعدد السكريد الشحمي واللقاح المقتول بالفورمالين للعصيات القولونية

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الخلاصة

أجريت هذه الدراسة لتقييم لقاح متعدد السكريد الشحمي واللقاح المقتول بالفورمالديهايد للعصيات القولونية وبجرعات مختلفة لتحديد الاستجابة المناعية للعصيات القولونية أيضا. وكذلك التغيرات المرضية بعد التلقيح الروتيني وبعد التحدي مع العصيات القولونية المسببة للإصابة، وتداخل هذه اللقاحات. استخدمت ثلاثمائة وخمسين فرخة تم تقسيم الأفراخ عشوائيا إلى 7 مجاميع تحتوي كل مجموعة 50 فرخة. ثلاث مجموعات لقحت عن طريق الرش باللقاح متعدد السكريد الشحمي وبمختلف الجرعات وثلاث مجموعات لقحت باللقاح المقتول عن طريق الحقن تحت الجلد وبجرعات مختلفة. كشفت النتائج ان لقاح متعدد السكريد الشحمي في جرعة 0.5 مل لكل طير أعطى ارتفاع معنوي ($P<0.05$) بعد التحدي مع العصيات القولونية المرضية بالمقارنة مع المجاميع الأخرى. حيث كانت نتائج المناعة الخلوية والخلطية والتغيرات المرضية افضل من باقي المجاميع.

الكلمات المفتاحية: لقاح العصيات القولونية، مرض النيوكاسل، دجاج اللحم، الاليزا.

Introduction

Escherichia coli belong to the Enterobacteriaceae family. It is a rod-shaped, Gram-negative, facultative anaerobic bacterium, The optimal growth of most *E. coli* strains occurs aerobically at 37°C and some exceptional strains can grow at temperatures up to 49°C (1). Poultry industry is excessively increased during last years. The growth of poultry industry is hampered by several factors including disease such as colibacillosis which is a known significant disease of poultry in the developing countries (2). *E. coli* infection is of great economic importance for poultry as a cause of various manifestations including salpingitis, peritonitis, perihepatitis, pericarditis and granuloma. These disease conditions are economically important to poultry producer because it causes morbidity, mortality, a lack of uniformity, decrease production

performance and increase condemnation in poultry flocks (3). LPS is a major component of the Gram-negative bacteria cell wall, and is capable of eliciting a plethora of effects including changes in body temperature, blood pressure and circulating leukocytes(4). Therefor effective vaccine to control these infections should be directed against antigenic determinants shared by all these serotypes. Serotype specificity of Gram-negative bacteria is determined by the O-polysaccharide side chains of bacterial LPS (5). The aims of current study to Isolation and characterization of a local isolate of *E. coli* from broilers infected with airsaccultis and evaluation the best vaccination program to protect the broilers against the pathogenic *E. coli* infection.

Materials and Methods

Isolation and identification *of E. coli* by using MacConkey and Eosine methylene blue (EMB) test according to the manufacture (Oxoid company. USA), according to Leboffe and Piercr (6), this test (Api- 20 E system) is used clinically for rapid identification of the bacteria isolates, also used PCR technique to confirmatory diagnosis of *E. coli* from infected cases according to the protocol company (Promega, Invitrogen, USA). The LPS vaccine was prepared from positive local isolate that infected with *E.coli* accoreding to Westaphal (7). As well as formaldehyde vaccine prepared according to Panigrahy (8). Three hundred and fifty chicks (Rose, Jordan Origin), were brought from AL-Baraka Hatchery–Baghdad. The chicks were divided randomly into 7 groups (50 chicks each) vaccinated with *E. coli* at day five as follow: G1: received LPS vaccine of *E. coli* through aerosal administration dose (0.15 ml from 3×10^7 CFU/ml). G2: received LPS vaccine of *E. coli* through aerosal administration dose (0.3 ml from 3×10^7 CFU/ml). G3: received LPS vaccine of *E. coli* through aerosal administration dose (0.5 ml from 3×10^7 CFU/ml). G4: received killed vaccine of *E. coli* through subcutaneous injection dose (0.15 ml from 3×10^7 CFU/ml). G5: received killed vaccine of *E. coli* through subcutaneous injection dose (0.3 ml from 3×10^7 CFU/ml). G6: received killed vaccine of *E. coli* through subcutaneous injection dose (0.5 ml from 3×10^7 CFU/ml). G7: control negative. Blood samples from each group were aspirated from jugular vein for determination of Ab titers against *E. coli* at day (20 and 35). Challenge by pathogenic *E. coli* in dose (3×10^8) intra tracheal inoculation at day 28 was took place.

Results and Discussion

The *E. coli* strain was isolated from the clinically affected broiler farms with chronic respiratory disease manifested the characteristic clinical signs of colibacillosis (respiratory signs, watery diarrhea, weakness, anorexia and weight loss etc.). *E. coli* which appeared a convex shape, dark pink color, and entire edges of colonies in the first culture on the MacConkey agar. The growth of *E. coli* on EMB agar was indicated by smooth, circular, black color colonies with metallic sheen This result is agreed with Bopp (9) Gram's staining revealed Gram-negative, short plump rod shaped bacteria, arranged in single, paired or in short chain under the microscope examination. The five basic sugars; dextrose, sucrose, lactose, maltose and mannitol were fermented by all the isolates of *E. coli* with the production of acid and gas. According to the result of API 20E test. The isolate was identified as *E.coli* with 99% confidence. DNA extracted from three *E. coli* isolates were used in the PCR assay. PCR primers for the *tuf* gene were used (*tuf* gene is found in all *E coli* strains) specific PCR band of 212 base pairs. All samples and the positive control showing the positive band with a size of 212 bp. Indicating that all of them are really *E.coli* isolates. The result of PCR agreed with Kawasaki (10). Fig. (1).

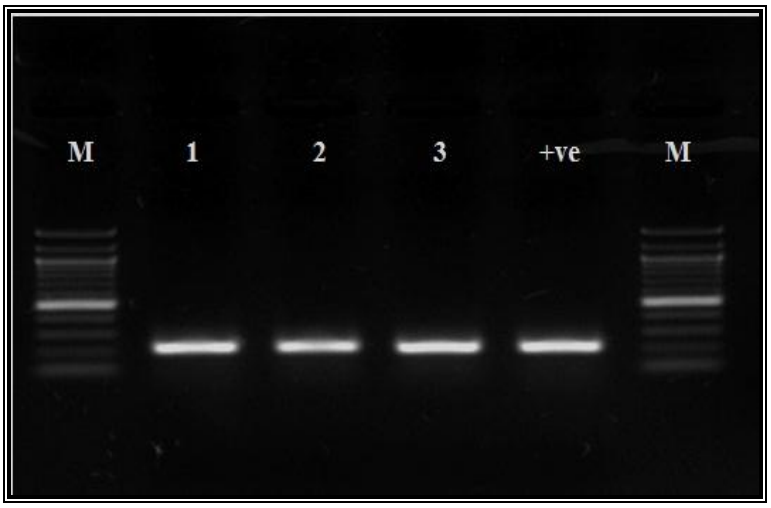

Fig. (1) Agarose gel electrophoresis of the PCR products amplified of the *tuf* gene of *E.Coli*. M: is 100 bp DNA marker, 1, 2 and 3 are the *E.coli* samples. And +ve: is the control positive

Avian colibacillosis caused by *E. coli* is a major health problems in poultry industry in Iraq (11). In this study, chicken clinically infected with colibacillosis manifested characteristics clinical signs of colibacillosis such as respiratory signs, watery diarrhea, anorexia, weakness and loss of body weight. Similar type of clinical signs in colibacillosis were also recorded by Kim (12). The results of the current study explained the presence of significant differences at level (P<0.05) among all groups in Ab titre against *E. coli* at (20 and 35) days old chicks. So at 20 days old chicks, the highest mean antibody level titre among the vaccinated groups was given by the sixth group which was (1.978) followed by the (third, fifth, second, fourth and first) groups, which were (1.763, 1.579, 1.476, 1.253 and 1.027) respectively, compared to the control group (seventh) which was (0.064) table (1). Whereas at day 35, the results reflected a significant increase at level (P<0.05) between all vaccinated groups in antibody titre against *E. coli* so the highest mean titre was given by group (sixth) which was (2.207) followed by (third, fifth, second, fourth and first) groups, which were (2.113, 1.786, 1.576, 1.456 and 1.297) respectively, compared to the control (seventh) group which was (0.911) table (1).

Table (1) The results of antibody titre against *E. coli* in the first experiment by ELISA test

Vaccine type	Dose	Group	20 day	35 day
Killed vaccine	0.15ml	1	1.027±0.019 F b	1.297±0.037 F a
	0.3ml	2	1.476±0.024 D b	1.576±0.02 D a
	0.5ml	3	1.763±0.026 B b	2.113±0.026 B a
LPS	0.15ml	4	1.253±0.024 E b	1.456±0.017 E a
	0.3ml	5	1.579±0.029 C b	1.786±0.018 C a
	0.5ml	6	1.978±0.022 A b	2.207±0.027 A a
Control		7	0.064±0.04 G b	0.911±0.028 G a

Means having different capital letters (in columns) and small letters (in rows) are significant difference * (P<0.05).

At day 20 the increment in antibody titre against avian pathogenic *E. coli* in the sixth group returns to the Lipopolysaccharide (LPS), known as endotoxin, which is the prominent constituent in the outer leaflet of outer membranes in most Gram-negative bacteria and plays an important role in membrane permeability, cell adhesion and stability (13). Intratracheal administration of LPS significantly affected secondary Ab responses, especially IgG and, to a minor extent, IgA responses. Pathogen-associated molecular patterns may affect dendritic cells, and consequently memory T-cell responses, or alternatively, may directly bind memory B cells, maintaining a nonantigen-specific form of memory (14). LPS showed direct activation of B-lymphocytes in vitro (15). LPS-activated B-lymphocytes produced antibodies of diverse specificities, mainly of the IgM type within a short of time period (16). On the other hand the increment in antibody titre against *E.coli* in the third group which explained the role of inactivated vaccine in dose 0.5 ml that elevated the antibodies against *E. coli* these results agree with Vaez zadeh, (17) who reported the increment of antibody titre against *E. coli* after vaccination with inactivated formaldehyde vaccine in one day old of newly hatched chicks. Also agree with Salim, (11) who mentioned the role of killed vaccine of *E. coli* with formalin to increase the serum antibody against E. coli at early days old. At day 35 the increase in antibody titre against *E. coli* was more than Ab titre at day 20 after challenge with virulent local strain of *E. coli* at day 28 that' interpret the role of vaccine especially LPS vaccine in dose 0.5 ml in the sixth group compared with the third group that vaccinated with killed vaccine, these findings agree with Peighambari (18) who mentioned that live-attenuated APEC vaccines or a low dose of virulent APEC confer a higher degree of cross-serogroup protection compared to killed vaccines. The serogroup-specificity of such vaccines has been inferred to be due to the dominance of responses to the lipopolysaccharide O antigen. As avian colibacillosis is caused by multiple APEC serotypes, requirement exists for broadly cross-protective vaccines (19). Production of the main serum-antibody isotypes, IgM and IgG, requires invasion of the *E. coli* antigen into the bloodstream. Exposure of turkeys to pathogenic *E. coli* leads to intestinal colonization without any pathological effects, but that stress enables these colonized bacteria to invade the digestive tract and penetrate into the bloodstream (20). Several experiments have been performed to prevent colibacillosis in turkeys by vaccination (21). Wild-type *E. coli,* administered via intratracheal or intramuscular (i.m.) routes, induced protective immunity in turkeys, whereas heat- or formalin-killed vaccines given without adjuvant did not afford this protection (22). Rapid clearance of virulent *E. coli* from the blood was enhanced by antibody-dependent phagocytosis in the liver (22). The results of current study explained presence of significant differences at level ($P<0.05$) among all vaccinated groups in mean thorasic air sac lesion scores at day 35, the lowest mean was recorded in (third, fifth, second and fourth) groups which were (0.5, 1, 1.25 and 1.75) but the sixth group did not record any score compared to first and control negative (seventh) which recorded (2.25 and 3) table (2). Also the mean heart lesion score was recorded significant decrease at level ($P<0.05$) among all vaccinated groups at day 35 the lowest mean was recorded in (sixth and third) group which was (0.25 and 0.25) followed by (fifth, second and fourth and first) which were (0.5, 0.75, 1 and 1.5) respectively, compared with control negative (seventh) which recorded (3.25) 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 lowest mean was recorded in (sixth and third) group which was (0.33 and 0.33) followed by (fifth, second and fourth and first) which were (0.66, 1, 1.33 and 1.66) respectively, compared to control negative (seventh) which recorded (2.33) table (2).

Table (2) Shown the Pathological changes of thoracic air sacs, heart and liver after infection with APEC strain for first experiment

Vaccine type	Dose	Group	Air sac score	Heart score	Liver score
Killed vaccine	0.15ml	1	2.25±0.8 A	1.5±0.95 AB	1.66±0.76 AB
	0.3ml	2	1.25±0.75 AB	0.75±0.75 AB	1±0.86 AB
	0.5ml	3	0.5±0.5 AB	0.25±0.25 B	0.33±0.28 A
LPS	0.15ml	4	1.75±1.03 AB	1±0.57 AB	1.33±0.57 AB
	0.3ml	5	1±0.57 AB	0.5±0.5 AB	0.66±0.57 AB
	0.5ml	6	0±0 B	0.25±0.25 B	0.33±0.28 B
Control negative		7	3±1.5 A	3.25±2.13 A	2.33±1.04 A

Means having different capital letters (in columns) are significant difference. * (P<0.05).

Systemic APEC infections are believed to arise from colonisation of the lower respiratory tract following inhalation of contaminated faecal dust (19). Colonisation of the air sacs is enhanced by suppression of muco-ciliary activity and other upper respiratory tract defenses resulting from concurrent infections and elevated ammonia levels in poultry houses. Avian air sacs are relatively a vascular structures lacking effective resident defense mechanisms (23). hence control of pathogens is thought to be reliant upon recruitment of heterophils and macrophages (24). The mode of translocation of APEC from the respiratory tract to the bloodstream is ill-defined. APEC can be found in the bloodstream as early as 3 hours after intra-air sac inoculation of naive birds (25) and the immune responses that constrain such spread are unclear. Systemic spread of APEC may be followed by sepsis or localized inflammation in survivors involving extensive heterophil infiltration in organs of the reticulo-endothelial system (26). The significant decrease in score lesion (air sac, liver and heart) in sixth group that's explained to role of LPS vaccine to protection the birds from challenge with APEC these finding agree with Dho-Moulin and Fairbrother, (27) we mentioned that the ability of immune serum induce by LPS to impair the net growth of an APEC, indicates that antibody-mediated killing in the presence of complement is not strictly dependent on the LPS (O-antigen). Proliferative responses to the APEC antigens were seen in mock-infected birds at three days pi and subsequent time points, suggesting non-specific stimulation or previous exposure to similar antigens (28). The separation of stimulated splenocytes with purified LPS from *E. coli* and obtained a similar pattern of responses (29).Transcripts encoding the pro-inflammatory cytokines IL-1β up-regulated in the lung during the early phase of infection. In contrast, an anti-inflammatory response was seen in the liver. Such strong responses are unusual in poultry, let alone in the liver, especially when the bacteria were present in large numbers and clinical signs of infection were present, consistent with this strong anti-inflammatory response (27). While the significant decrease in mean of score lesion in third group agree with Hanan (30) reported that vaccinated with inactive E. coli by formalin protected the vaccinated bird from challenge with APEC at 3 weeks of age and slightly recorded pathological changes.

References

1. Fotadar, U.; Zaveloff, P. & Terracio, L. (2005). Growth of Escherichia coli at elevated temperatures. J. Basic Microbiol., 45: 403-404.
2. Jordan, F. T. W.; Williams, N. J.; Wattret, A. & Jones, T. (2005). Observations on salpingitis, peritonitis and salpingoperitonitis in a layer breeder flock. Vet. Rec., 157: 573-577.
3. Gross, W. B. (1966). Electrocardiographic changes in *Escherichia coli* infected birds. Am. J. Vet. Res., 27: 1427-1436.
4. Nakamura, T.; Ebihara, I. & Nagaoka, I. (1992). Activated peripheral blood mononuclear cells in IgA nephropathy express platelet-derived growth factor. J. Lab Clin. Med., 120: 212-221.
5. Galanos, C. & Lu¨deritz, O. (1984). Lipopolysaccharide: properties of an amphipathic molecule. In Hand Book of Endotoxin. 1. Chemistry of Endotoxin, PP. 46-58. Edited by E. Th. Rietschel. Amsterdam: Elsevier.
6. Leboffe, M. J. & Pierce, B. E. (2005). A Photographic Atlas for the Microbiology Laboratory, 3rd ed., Morton Publishing Co. ISBN: 0-89582-656-9.
7. Westphal, O. & Jann, K. (1965). Bacterial LPS extraction with phenol water and further application of the procedure, PP.83-91. In: R. L. whistter (ed), Methods in carbohydrate chemistry. Academic Press, Inc., New York.V.5.
8. Panigrahy, B.; Gyimah, J. E.; Hall, C. F. & Williams, J. D. (1984). Immunogenic potency of an oil-emulsified Escherichia coli Bacterin. Avian Dis., 28:475-481.
9. Bopp, C. A.; Brenner, F. W.; Fields, P. I.; Wells, J. G. & Strockbine, N. A. (2003). Escherichia, Shigella and Salmonella, PP. 654-671. In Murray, P. R., E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), Manual of clinical microbiology, vol. 1. ASM Press.
10. Kawasaki, S.; Fratamico, P. M.; Horikoshi, N.; Okada, Y.; Takeshita, K.; Sameshima, T. & Kawamoto, S. (2009). Evaluation of a multiplex PCR system for simultaneous detection of Salmonella spp., Listeria monocytogenes, and Escherichia coli O157:H7 in foods and in food subjected to freezing. Foodborne Pathogens and Disease, 6 (1): 81- 89.
11. Salim, S. A. (2014). Preparation and evaluation of different types of avian pathogenic E-coli Antigen in broiler chicken. MSc. Thesis, College of veterinary medicine, University of Baghdad.
12. Kim, H. J.; Kong, M. I.; Clung, U. K.; Kim, H. J.; Kang, M. I. & Clung, U. I. (1996). Survey of enteric diseases in Chickens. Korean J. Vet. Res., 36: 1007-1012.
13. Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev., 67:593-656.
14. Bernasconi, N.; Onai, N. & Lanzaveccia, A. (2003). A role for toll like receptors in acquired immunity: Upregulation of TLR9 by PCR triggering in naive B cells and constitutive expression in memory B cells. Blood, 101:4500-4504.
15. Andersson, J.; Sjöberg, O. & Möller, G. (1972). Induction of immunoglobulin and antibody synthesis in vitro by lipopolysaccharides. Eur. J. Immunol., 2: 349-353.
16. Andersson, J.; Coutinho, A. & Melchers, F. (1978). Stimulation of murine B lymphocytes to IgG synthesis and secretion by the mitogens lipopolysaccharide and lipoprotein and its inhibition by anti-immunoglobulin antibodies. Eur. J. Immunol., 8: 336-343.

17. Vaez zadeh, F.; Esmaily, F. & Sharifi-Yazdi, M. K. (2004). Protective immune responses induced in chickens by outer membrane proteins extracted from different strains of Escherichia coli. Iran. J. Allergy Asthma Immunol., 3: 133-137.
18. Peighambari, S. M.; Hunter, D. B.; Shewen, P. E. & Gyles, C. L. (2002). Safety, immunogenicity, and efficacy of two Escherichia coli cya crp mutants as vaccines for broilers. Avian. Dis., 46(2): 287-297.
19. Dziva, F. & Stevens, M. P. (2008). Colibacillosis in poultry: unraveling the molecular basis of virulence of avian pathogenic Escherichia coli in their natural hosts. Avian Pathol., 37(4): 355-366.
20. Leitner, G. & Heller, E. D. (1992). Colonisation of *Escherichia coli* in young turkeys and chickens. Avian Dis., 36: 211-220.
21. Kwaga, J. K.; Allan, B. J.; van der Hurk, J. V.; Seida, H. & Potter, A. (1994). A carAB mutant of avian pathogenic Escherichia coli serogroup O_2 is attenuated and effective as a live oral vaccine against colibacillosis in turkeys. Infect. Immun., 62:3766-3772.
22. Zhao, L.; Gao, S.; Huan, H.; Xu, X.; Zhu, X.; Yang, W.; Gao, Q. & Liu, X. (2009). Comparison of virulence factors and expression of specific genes between uropathogenic *Escherichia coli* and avian pathogenic *E. coli* in a murine urinary tract infection model and a chicken challenge model. Microbiology, 155(Pt 5): 1634-1644.
23. Ficken, M. D.; Edwards, J. F. & Lay, J. C. (1987). Effects of Newcastle disease virus infection on the binding, phagocytic, and bactericidal activities of respiratory macrophages of the turkey. Avian Dis., 31: 888-894.
24. Toth, T. E. & Siegel, P. B. (1986). Cellular defense of the avian respiratory tract: paucity of free-residing macrophages in the normal chicken. Avian Dis., 30(1): 67-75.
25. Pourbakhsh, S. A.; Boulianne, M.; Martineau-Doize, B. & Fairbrother, J. M. (1997). Virulence mechanisms of avian fimbriated Escherichia coli in experimentally inoculated chickens. Vet. Microbiol., 58: 195-213.
26. Vandekerchove, D.; de Herdt, P.; Laevens, H. & Pasmans, F. (2004). Colibacillosis in caged layer hens: characteristics of the disease and the aetiological agent. Avian Pathol., 33(2):117-125.
27. Dho-Moulin, M. & Fairbrother, J. M. (1999). Avian pathogenic Escherichia coli (APEC). Vet. Res., 30 (2/3): 299-316.
28. Ghosh, R. C.; Hirpurkar, S. D. & Suryawnshi, P. R. (2006). Concurrent colibacillosis and infectious bursal disease in broiler chicks. Indian Vet. J., 83: 1019-1020.
29. El-Sukhon, S. N.; Asad, M. & Al-Attar, M. (2002). Studies on the bacterial etiology of air sacculitis of broiler in northern and middle Jordan with special reference to *Escherichia coli*, Ornithobacterium rhinotracheale and Bordetella. Avian Dis., 46: 605-612.
30. Hanan A. A.; Hoda, M. M. & Ghada, M. E. (2012). Studies on Vaccination of Turkey against *Escherichia coli* Infection. Global Veterinaria 8 (6):601-604.