UNIVERSITI PUTRA MALAYSIA

EFFICACY OF INACTIVATED AVIAN PATHOGENIC *ESCHERICHIA COLI* AGAINST THE BACTERIAL INFECTIONS IN BROILER CHICKENS

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EFFICACY OF INACTIVATED AVIAN PATHOGENIC *ESCHERICHIA COLI* AGAINST THE BACTERIAL INFECTIONS IN BROILER CHICKENS

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It is hereby certified that we have read this project paper entitled "Efficacy of Inactivated Avian Pathogenic *Escherichia coli* against the Bacterial Infections in Broiler Chickens", by Wendy Yong Wai Kheng and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of requirement for the course VPD 4999-Final Year Project.

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KEBERKESANAN *ESCHERICHIA COLI* PATOGENIK AVIAN YANG TIDAK AKTIF TERHADAP JANGKITAN BAKTERIA DALAM AYAM PEDAGING

Oleh

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Escherichia coli Patogenik Avian (APEC) menyebabkan colibacillosis dalam ayam dan ia menunjukkan pelbagai keadaan klinikal seperti colisepticemia dan selulitis. Tujuan kajian ini adalah untuk mengenalpastikan keberkesanan APEC yang tidak aktif sama ada sebagai tunggal atau kombinasi gen berkaitan virulen (VAG) 5 dan 6 terhadap jangkitan bakteria dalam ayam pedaging. Lapan puluh empat ekor ayam berumur satu hari dibahagi sama kepada tujuh kumpulan. Pada umur satu hari, setiap ayam dalam kumpulan 1 dan 4 telah diberi suntikan bawah kulit dengan APEC yang tidak aktif dengan VAG 5. Kumpulan 2 dan 5 dengan VAG 6, dan kumpulan 3 dan 6 dengan gabungan VAG 5 dan VAG 6. Kumpulan 7 tidak diberi suntikan kerana ia adalah kumpulan kawalan. Pada umur 14 hari, boster telah diberikan kepada kumpulan 4, 5 dan 6. Pada umur 28 hari, ayam dalam setiap kumpulan telah dibahagikan kepada kumpulan dicabar dan tidak dicabar. Kumpulan dicabar telah diinokulasi (0.1 mL) dengan VAG 6 (10^8

cfu/mL) sama ada melalui intramuskular atau laluan intranasal. Pada hari ke 35, semua ayam dikorbankan. Hati dan limpa diambil untuk mengenalpastikan kehadiran bakteria, manakala hati dan trakea untuk histopatologi. Kajian menunjukkan dua ayam dari kumpulan kawalan terbantut pertumbuhan, dehidrasi dan kurang selera makan selepas dicabar dengan suntikan intramuskular, dan menunjukkan lesi kasar perihepatitis dan pericarditis. *E. coli* dapat diasingkan hanya daripada satu sampel dari kumpulan yang sama. Histologi menunjukkan hepatitis teruk dengan hepatosit nekrosis dan degenerasi yang teruk, dan tracheitis dengan nekrosis dan degenerasi yang teruk. Sebaliknya, hepatitis ringan dengan hepatosit nekrosis dan degenerasi ringan, dan tracheitis dengan nekrosis dan degenerasi ringan telah direkod dalam kumpulan ayam tanpa tanda klinikal. Berdasarkan tanda klinikal, lesi dan hasil pengasingan bakteria, APEC tidak aktif sama ada sebagai tunggal atau kombinasi dengan VAG 5 dan 6 memberikan perlindungan yang lebih baik terhadap jangkitan bakteria di dalam ayam pedaging berbanding dengan kumpulan tidak diinokulasi. Kesimpulannya, APEC tidak aktif sama ada sebagai tunggal atau kombinasi dengan VAG 5 dan 6 adalah berkesan dan mampu memberikan perlindungan dicabar dengan VAG 6.

Kata kunci: *Escherichia coli* patogenik avian (APEC), tidak aktif, gen berkaitan virulen (VAG), ayam pedaging

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, Universiti Putra Malaysia in partial fulfillment of the course VPD 4999 – Project

EFFICACY OF INACTIVATED AVIAN PATHOGENIC *ESCHERICHIA COLI* AGAINST THE BACTERIAL INFECTIONS IN BROILER CHICKENS

By

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2016

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Co-supervisor: Assoc. Professor Dr. Zunita Zakaria

Avian Pathogenic *Escherichia coli* (APEC) causes colibacillosis in poultry, which it manifests various clinical conditions such as colisepticemia and cellulitis. This objective of this study was to determine efficacy of inactivated APEC either as single or combination with virulent associated gene (VAG) 5 and 6 against the bacterial infection in broiler chickens. Eighty-four day-old broiler chickens were equally divided into seven groups. On day 1, groups 1 and 4 were inoculated subcutaneously with inactivated APEC with VAG 5. Groups 2 and 5 with VAG 6 and groups 3 and 6 with combination of VAG 5 and VAG 6. Group 7 was not inoculated as it was acted as control group. On day 14, booster was given to groups 4, 5 and 6. On day 28, the chickens in each group were divided into the challenge and non-challenge groups. Chickens in the challenge groups were inoculated (0.1 mL) with VAG 6 (10^8 cfu/mL) either via intramuscular or intranasal routes. On day 35, all the chickens were sacrificed. The livers and spleens were collected for bacterial identification, while livers and trachea for histopathology.

The study showed that two chickens from the control group were stunted growth, dehydrated and inappetence after challenged intramuscularly, and showed gross lesions of perihepatitis and pericarditis. *E. coli* was isolated from only one sample of the same group. Histologically, severe hepatitis with severe necrosis and degeneration of the hepatocytes, and severe tracheitis with severe necrosis and degeneration were recorded. In contrast, mild lesions of hepatitis with mild necrosis and degeneration of hepatocytes, and mild tracheitis with mild necrosis and degeneration were recorded in groups of chickens without clinical signs. Based on the clinical signs, lesions and bacterial culture, the inactivated APEC either as single or combination with VAG 5 and 6 gives better protection against the bacterial infection in broiler chickens compared to non-inoculated group. In conclusion, inactivated APEC either as single or combination with VAG 5 and 6 was effective and could provide protection against VAG 6 challenged.

Keywords: Avian Pathogenic *Escherichia coli* (APEC), inactivated, virulent associated gene (VAG), broiler chickens

1.0 INTRODUCTION

Avian Pathogenic *Escherichia coli* (APEC) belongs to the extraintestinal pathogenic group of *E. coli* (ExPEC), and it is the etiologic agent of colibacillosis. Colibacillosis, includes multiple extra–intestinal diseases often respiratory, leading to localized or systemic infection such as colisepticaemia, coligranuloma, chronic respiratory disease, coliform cellulitis, peritonitis, salphingitis, panophthalmitis, yolk sac infection and enteritis (Barnes *et al*., 2008). Localized or systemic infections depending on the strain of *E. coli*, age and the gender of the chicken, as well as the immunologic status and the presence of predisposing environmental conditions (Dizva & Stevens, 2008). Colibacillosis is therefore responsible for significant economic losses to the poultry industry due to decrease in growth rate and egg production, condemned in carcasses, mortality and cost of treatment and production.

According to Moriel *et al.* (2010), avian colibacillosis is difficult to control because of two major issues which are limited reliable methods to identify the causative strains of *E. coli* and lack of effective vaccine. These factors are due to variable characteristics of APEC strains preventing the identification of common properties, which could be the basis for diagnostic methods and vaccination. Diagnostic methods such as serotyping and genotyping are used to identify APEC strains. Although serotyping remains the most frequently used method in laboratories, it only identifies limited number of APEC strains (Schouler *et al*.,

2012). While virulence genotyping is to detect genes encoding virulence factors by using PCR amplification.

In the last decade, there is a growing concern of the use of antimicrobial drugs in veterinary medicine especially in the food production animals. Excessive and prolonged usage of antimicrobial drugs may compromise human health if resistant bacteria develop in food production animals are transferred to humans via food chain or the environment (Vuuren, 2003). Therefore, instead of using antimicrobial drugs, bacterial infection can be controlled by using a different approach which is vaccine. One of the earliest attempts to control colibacillosis by vaccination was by Gross (1957), where he used formalin inactivated O2:K1 and O78:K80 serotypes of *E. coli* as vaccines (Gross, 1957). Overall, the inactivated vaccines provided protection against homologous challenge only. Several factors were shown to play a role in determining the efficacy of an inactivated APEC vaccine namely the serogroup or serotype of the *E. coli* included in the vaccine, the type of adjuvant used, the method used to inactivate the bacteria, the route and frequency of administration, and the age of the chickens at the time of vaccine administration (Ghunaim *et al*., 2014).

Hence, the hypothesis of this study were inactivated APEC could provide protection against APEC challenge. Inactivated APEC combination of VAG 5 and VAG 6 could provide better protection against APEC challenge when compared to the single VAG 5 or VAG 6. Booster of inactivated APEC either with VAG 5, 6 or

combination of VAG 5 and 6 could provide protection against APEC challenge infection when compared to non-booster group.

The objective of this study was to determine the efficacy of inactivated APEC either as single or combination of APEC with VAG 5 and VAG 6 against the bacterial infection in broiler chickens.

2.0 LITERATURE REVIEW

2.1 *Escherichia coli*

2.1.1 Classification

Escherichia is the type genus which belongs to the family Enterobacteriaceae. The Enterobacteriaceae family is composed of organisms that can grow aerobically or anaerobically and utilize simple carbon and nitrogen. *E. coli* is the type species of the genus Escherichia, and it occurs most commonly and is the most important pathogen compare to other species in the genus (Barnes *et al*., 2008).

2.1.2 Morphology

Escherichia coli is a gram negative, non-acid-fast, uniform staining, non-spore-forming rods, normally has the size of 2-3 x 0.6 µm. *E. coli* which are grown in the culture are more variable in shape and size (Barnes *et al*., 2008).

According to Barnes *et al*., (2008), *E. coli* colonies are generally low, convex and smooth on agar plates which are incubated for 24 hours at 37^{o}C, but different agar plates can produce different types of colony morphology. *E. coli* which are grown on MacConkey agar produce bright pink colonies and are surrounded by a precipitate. Eosin-methylene blue (EMB) agar produces colonies which have a dark green-black metallic sheen. Besides, the *E. coli* colonies are yellow on tergitol-7 agar. *E. coli* colony morphology may vary, but they are usually 1-3 mm in diameter with granular structure and an entire margins. *E. coli* can also produce mucoid colonies which are raised, large, appear wet, and are sticky when probed.

For APEC, haemolysis on blood agar is not common which is different from mammalian pathogenic *E. coli* which occurrence of haemolysis is frequent (Barnes *et al*., 2008).

2.1.3 Biochemical Properties

E. coli produces acid and gas from the fermentation of glucose, maltose, mannitol, xylose, glycerol, rhannose, sorbitol, and arabinose, but not dextrin, starch, or inositol. *E. coli* produces indole, a positive methyl red reaction, and reduces nitrate to nitrite. *E. coli* is also catalase positive but oxidase negative. It does not produce hydrogen sulfide, and it is also citrate and urease negative (Barnes *et al*., 2008).

2.1.4 Antigenic Structure and Toxins

The antigens of *Escherichia coli* are O (Somatic) antigen, H (Flagellar) antigen, K (Capsular) antigen, F (Pilus) antigen and toxins. According to Kauffmann scheme, serotypes of *E. coli* are classified based on O antigen, H antigen and K antigen (Lior, 1996). However, in most serologic typing schemes, only the O and H antigens are considered, e.g., O157;H7. Serogroup of *E. coli* is determined by O antigen while serotype is determined by H antigen. Fimbrial (pilus) antigens are included in serotyping when considered important.

O antigen is the antigenic portion of lipopolysaccharide (LPS) in the cell wall, which is also known as endotoxin. LPS is a polysaccharide-phospholipid complex that is released when the cell undergoes lysis. H antigens are proteins found in

the different types of flagellin that comprise of flagella. K antigens are polymeric acids containing 2% reducing sugars. K antigens are associated with virulence, are on the cell surface which will interfere with O agglutination. P antigens take part in attaching to the cells. Toxins produced by APEC are much less toxigenic compared to the toxins produced by pathogenic *E. coli* in mammals and human beings.

2.2 Avian Pathogenic *E. coli*

Avian pathogenic *E. coli* is the aetiology of colibacillosis in birds. Unlike other pathogenic groups of *E. coli*, no single trait or group of traits defines the APEC pathotype (Dziva and Stevens, 2008). Therefore, phenotypic and genotypic characteristics are used for strain classification. Phenotypic characteristics of APEC are identified using serotyping while genotyping characteristics are identified using genetic or molecular methods.

2.2.1 Strain Classification

Serotyping

Serotyping method is to determine O and H antigens of APEC. Determination of both O and H antigens are by agglutination using O and H antisera. According to *Schouler et al*. (2012), serotyping remains the most widely used diagnostic method in laboratories despite it only allows the identification of a certain number of APEC strains.

Genotyping

By using multiplex polymerase chain reaction (PCR), several characteristics of APEC can be identified by detecting genes that encoding virulence factors. According to study done by Ewers *et al.* (2005), strains were classified as APEC if the *E. coli* from diseased birds harboured at least four of the eight following genes which are P fimbriae (*papC*), temperature-sensitive haemagglutinin (*tsh*), aerobactin (*iucD*), iron repressible protein (*irp2*), vacuolating autotransporter protein (*vat*), enteroaggregative toxin (*astA*), increased serum survival protein (*iss*) and colicin plasmid operon genes (*cva/cvi*).

2.2.2 Virulence Factors

Colonization Factors

Fimbriae are proteinaceous filaments or appendages expressed on the surface of bacteria that are believed to mediate adherence to host cells (Dziva and Stevens, 2008). There are different types of fimbrial adhesins. P fimbriae (*papC*) is one of the fimbrial adhesins which would be important in APEC's colonization of host despite its pathogenesis in avian colibacillosis is unclear (Babai *et al.,* 2000). Pourkashsh et al. (1997) reported that P fimbriae colonize lower respiratory tract and other internal organs but not trachea. F1 fimbriae are expressed instead during the initial colonization of trachea epithelial cells.

Temperature-sensitive haemagglutinin (*tsh*) is also involved in colonization, especially in the early stages of infection including colonization of air sacs but not

subsequent generalized infection (Dozois et al., 2000). Besides, *tsh* can adhere to red blood cells and also bind to extracellular matrix proteins (Kostakioti and Staphopoulos, 2004).

Iron Acquisition Systems

The ability of APEC to sequester iron from body fluids due to various iron-acquisition mechanisms. A study done by Dho and Lafont (1984) has linked the lethality of day-old chicks with this characteristics. Virulent strains of *E. coli* express genes such as aerobactin (*iuD*) for synthesis of the hydroxamate siderophore and for ferric aerobactin uptake respectively which both of the genes are involved in iron-acquisition mechanism (Warner *et al.,* 1981). Iron-repressible protein (*irp2*) had been detected by PCR in more than 65% of strains isolated from cases of avian colibacillosis (Jansen et al., 2001).

Serum Resistance Mechanism

A region on colicin plasmids (*cva/cvi*) harbouring *traT* and *iss* genes which encode outer membrane proteins, and they are involved in serum resistance (Wooley et al., 1993). They have the ability to resist complement-mediated lysis and opsophagocytosis which play an important role in APEC virulence.

Toxins

Vacuolating autotransporter toxin (*vat*) which is encoded by the *vat* gene, is commonly found in APEC. It causes cytotoxic effects in cultured cells similar to

those caused by *Helicobacter pylori* VacA toxin. Enteroaggregate toxin (astA) is also reported in APEC strains (Jassen et al., 2001).

2.3 Avian Colibacillosis

Colibacillosis refers to any localized or systemic infection caused entirely or partly by avian pathogenic *Escherichia coli* (APEC). It is the most common infectious bacterial disease of poultry and are responsible for significant economic losses (Barnes *et al*., 2008).

2.3.1 Epidemiology

All birds are susceptible to colibacillosis especially young birds. Factors increasing susceptibility of colibacillosis occurrence include damaged skin or mucosal barriers, impaired mononuclear-phagocytic system, immunosuppression caused by nutrition deficiencies or viral infection and unfavourable environment such as poor ventilation, high stocking density and temperature fluctuation (Barnes *et al*., 2008).

2.3.2 Clinical Signs

According to Barnes *et al*. (2008), colibacillosis produces various clinical signs depending on the specific type of disease produced by *E. coli*. Localized infections generally result in milder clinical signs than systemic diseases. Clinical sign such as coliform cellulitis is usually only detected when the birds are processed. Lameness and retarded growth are also the clinical signs which are caused by skeletal lesions as a sequel of sepsis. Faeces produced by the infected birds are

usually white to yellow urates as a result of anorexia and dehydration. Young birds with distended abdomen often have omphalitis and infected yolk sacs. Birds with colisepticemia are often terminally moribund, inactive, inappetence, dehydrated and eventually dead.

2.3.3 Pathology

Colibacillosis manifests various pathological lesions ranging from coliform omphalitis, coliform cellulitis, swollen head syndrome, diarrhoea, salphingitis and orchitis caused by localized infections and colisepticemia caused by systemic infections.

Colisepticemia is the presence of virulent *E. coli* in the blood stream which progresses through stages like acute septicaemia, subacute polyserositis and chronic granulomatous inflammation (Cheville and Arp, 1978). Characteristic features of colisepticemia at necropsy are tissues that develop a green discolouration and characteristic odour. The bursa of Fabricius is often atrophic or inflamed as a result of colisepticemia, and it should not be interpreted as infectious bursal disease (Nakamura *et al*., 1986). Pericarditis followed by fibrinous exudate as disease progresses is also common and a characteristic of colisepticemia. *E. coli* can also gain access to the blood circulation from damaged respiratory mucosa. Predisposing agents for respiratory-origin colisepticemia are infectious bronchitis virus, Newcastle disease virus, mycoplasmas and ammonia. Infected air sacs are thickened and often have caseous exudate on the respiratory surface (Barnes *et al*., 2008). Meanwhile, edema and heterophil infiltration can

be seen microscopically for lung and trachea in early infection. Later, macrophages, giant cells, fibroblast proliferation, necrotic heterophils in caseous exudate are common.

E. coli can also gain access through damaged intestinal mucosa caused by predisposing agent such as haemorrhagic enteritis virus (Newberry *et al*., 1993). The affected birds are in good appearance and often have full crops. The most characteristic lesions are congestion or green discolouration of liver, enlarged and spleen, and congested muscles. Microscopically the liver has fibrin present in the sinusoids. Besides, areas of acute necrosis can be seen initially, but evolve into granulomatous hepatitis with time in survivors. While for spleen under microscope, it is congested with proteinaceous fluid in sinuses and has multifocal necrosis containing intralesional bacteria (Barnes *et al*., 2008).

2.3.4 Diagnosis

Diagnosis is based on isolation and identification of *E. coli* from lesions typical of colibacillosis. Multiplex PCR can be done to distinguish pathogenic isolates from non-pathogenic *E. coli* (Barnes *et al*., 2008).

2.3.5 Treatment

Fluoroquinolones was proved efficacious in treating colibacillosis in poultry (Glisson *et al*., 2004). However, before selecting antimicrobial for treatment, antimicrobial susceptibility test should be done before treatment so as to avoid using ineffectively. Antimicrobial drugs have been used extensively for bacterial

infection causing antimicrobial resistance to develop progressively which will eventually lead to lose of efficacy of the antimicrobials (Sojka and Carnaghan, 1961).

2.3.6 Vaccine

An effective vaccine against APEC is crucial to control APEC infections in today's modern intensive broiler industry (Ghunaim *et al*., 2014). Inactivated vaccine is produced by inactivating APEC with either heat, acetone, alcohol or formalin. The inactivated vaccines can only provide protection against homologous challenge of *E. coli* only which means the chickens are not protected when they are challenged by *E. coli* from other serogroups (Ghunaim *et al*., 2014). While live-attenuated vaccine contains either live avirulent or diluted virulent strains. Studies show live-attenuated vaccines are able to induce immunization against heterologous *E. coli*. However, it required thorough investigation under field conditions (Ghunaim *et al*., 2014).

3 MATERIALS AND METHODS

3.1 Bacterial Isolates

Two isolates were characterized as APEC by PCR, APEC-UPM 1304 which is VAG 5 has five virulence genes, while APEC-UPM 1305 which is VAG 6 has six virulence genes (Table, 1).

Table 1: Virulence associated genes (VAG) of the two *E. coli* isolates of Malaysia

Virulence associated genes / APEC Isolates	*vat*	*iss*	*irp2*	*papC*	*iucD*	*cva/cvi*
APEC-UPM 1304 (VAG 5)	/	/	/	-	/	/
APEC-UPM 1305 (VAG 6)	/	/	/	/	/	/

3.2 Bacterial Culture and Identification

The bacterial stocks APEC-UPM 1304 and APEC-UPM 1305 were streaked onto fresh blood agar plates and were kept in the incubator overnight at 37^{o}C. Biochemical tests including triple sugar ion (TSI) test, sulphide indole motility (SIM) test, Simmons Citrate agar, Chinstensen's urea agar, Methyl Red-Voges Proskauer (MRVP) broth and Oxidase test and Brain Heart Infusion Medium (BHI) were performed to confirm the identity of the bacterial colonies on the blood agar plates (Barnes *et al.,* 2008). A single colony of *E. coli* was transferred from

the blood agar plate to BHI and was incubated overnight in a shaker rotating at 200 rpm at 37°C to obtain bacterial inoculum. Serial dilution was performed on the bacterial inoculum (VAG 5 and VAG 6) to obtain a concentration of 10^{11} cfu/mL using McFarland standards (Olarinmoye *et al.,* 2013).

3.3 Bacterial Inactivation

Formalin was added into bacterial inoculums to the final concentration of 2.5% formalin. The mixture of bacterial inoculums and formalin was mixed by vortex and was kept in the incubator at 37°C for 2 hours. 100µl of the mixture was spread onto fresh blood agar plate to grow. No bacterial growth indicates the bacteria were fully inactivated (Gross, 1957).

3.4 Preparation of Bacterial Inoculums

Aluminium potassium sulphate adjuvant (Alum, 100 gm) was added into 1 litre of R.O. water. Both the inactivated bacterial inoculums were combine with alum at the ratio of 1:10. The mixture was emulsified with a homogenizer until both the inoculums and adjuvant were properly mixed. The bacterial inoculums with alum were kept in refrigerator at 4°C.

3.5 Experimental Design

A total of 84 broiler day-old chicks were equally divided into 7 groups of 12 chickens in each group. All the chickens were fed *ad libitum* commercial feed and water throughout the trial. The chickens were monitored for abnormal clinical signs every day throughout the trial.

On day 1, groups 1 and group 4 were inoculated with 0.1 mL of inactivated VAG 5; group 2 and 5 were inoculated with 0.1 mL of inactivated VAG 6; group 3 and 6 were inoculated with 0.1 mL of combination of inactivated VAG 5 and VAG 6. On day 14, group 4, 5 and 6 were given booster of VAG 5, VAG 6 and combination of VAG 5 and 6 respectively.

On day 28, eight chickens from each group were challenged with 0.1 mL of VAG 6 (10^8 cfu/mL) either via intramuscular (n=4) or intranasal (n=4) route. On day 35, all the chickens were sacrificed and necropsy was conducted. Livers and spleens were collected for bacterial isolation and identification, while livers and trachea were collected for histopathology.

The use of chickens in the study was approved by Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia on December 21st, 2015 with reference number UPM/IACUC/FYP.2015/FPV.042.

3.6 Bacterial Isolation and identification

Samples of livers and spleen were collected from all the chickens after necropsy. The surface of the organs were sterilized with a heated scalpel blade, and scalpel

blade was used to cut into the sterilized surface of the organs. A sterile cotton swab was used to swab on the inner cut surface, and streaked on MacConkey agar plate. The MacConkey agar plates were incubated for 24 hours (Barnes *et al*., 2008). If there was presence of colonies growth on MacConkey agar, each colony was sub-cultured on blood agar plates. Each type of colonies grown on the blood agar was examined under the microscope with Gram stain. Only the gram negative bacillus was tested with biochemical test which were triple sugar iron (TSI), sulfide indole motility (SIM), urea, citrate, Indole test and oxidase tests to identify *E. coli*.

3.7 Necropsy and Histology

General appearance and organs of all the chickens were examined. Samples of livers and tracheas were collected and fixed in 10% formalin. Samples of livers and tracheas were trimmed, processed, embedded, sectioned (Ross and Pawlina, 2006) and stained with hematoxylin and eosin (HE) (Fischer *et al.,* 2008).

4 RESULTS

4.1 Clinical Signs

All the chickens from all groups were healthy throughout the experiment either before or after challenge, except that there were two chickens from the control group which were challenged with VAG 6 intramuscularly were stunted, lame, inactive and dehydrated (Figure, 1).

Figure 1: Two of the chickens from control group (IM) were stunted and dehydrated at day 2 post challenged via intramuscular route.

Gross Lesions

Most of the chickens were normal except the two stunted chickens from control group which were challenged with VAG 6 intramuscularly. These chickens showed gross lesion of fibrinous perihepatitis and pericarditis at day 7 post challenge (Figure, 2).

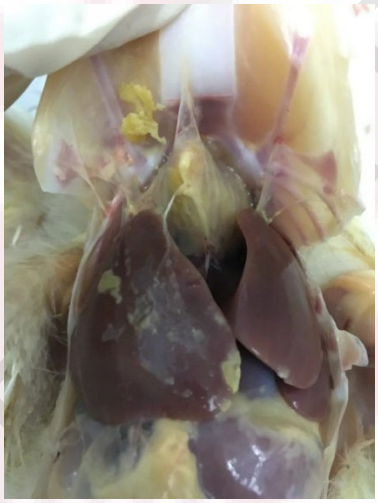

Figure 2: Chicken from the control group with gross lesion of fibrinous perihepatitis and pericarditis at day 7 post challenge via intramuscular route.

4.2 Bacterial Isolation and Identification

Samples of liver and spleen were negative for *E. coli*, except there was growth from the samples of one chicken from the control group challenged with VAG 6 via intramuscular route. It was oxidase negative, H_2S negative, indole positive, produced gas, citrate and urea negative which was confirmative of *E. coli*.

4.3 Histopathology

All samples of liver from all groups of the chickens either challenged or not challenged by VAG 6 showed normal to mild lesions such as mild inflammation, necrosis and degeneration (Figures, 3 and 4). However, liver of the chickens which had the clinical signs showed severe hepatitis with marked inflammatory cells infiltration and hepatic necrosis and degeneration (Figure, 5). Samples of trachea from all groups showed normal to mild lesions with mild inflammation, necrosis and degeneration (Figures, 6 and 7). The trachea from the chickens which had clinical signs showed severe tracheitis with marked infiltration of inflammatory cells at the submucosa and severe necrosis and degeneration (Figure, 8).

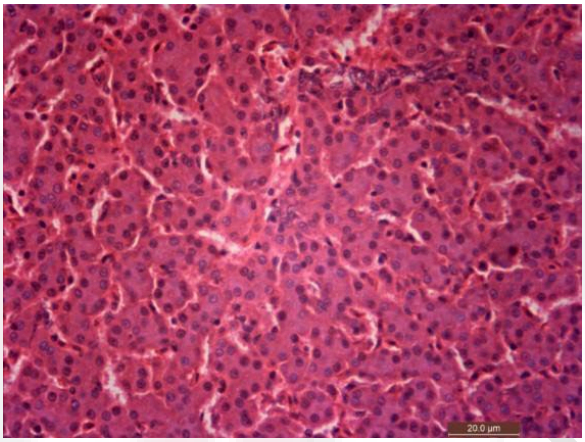

Figure 3: Liver of chickens from group 5 showed mild hepatitis with mild hepatic necrosis and degeneration. HE. Bar= 20 µm

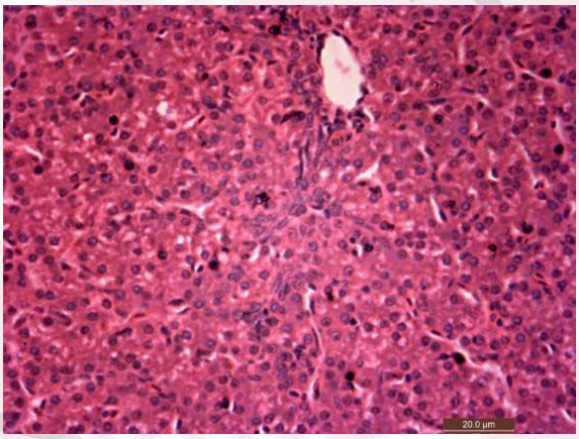

Figure 4: Liver of chickens from group 6 showed mild hepatitis with mild hepatic necrosis and degeneration. HE. Bar= 20 µm

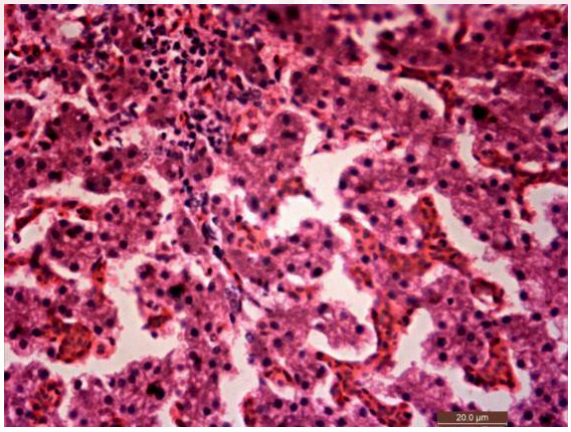

Figure 5: Liver of the chickens which had the clinical signs showed severe hepatitis with marked inflammatory cells infiltration and hepatic necrosis and degeneration. HE. Bar= 20 µm

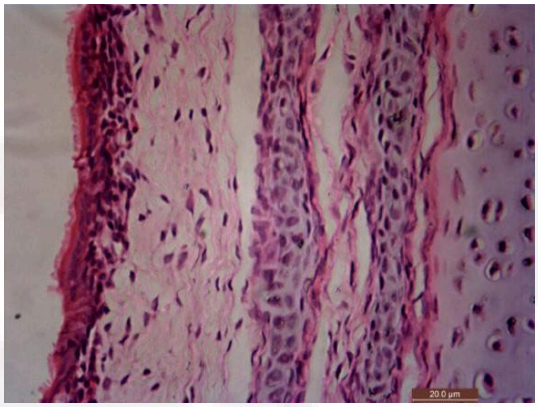

Figure 6: Trachea of chickens from group 5 showed normal to mild tracheitis with mild epithelial cells necrosis and degeneration HE. Bar= 20 µm

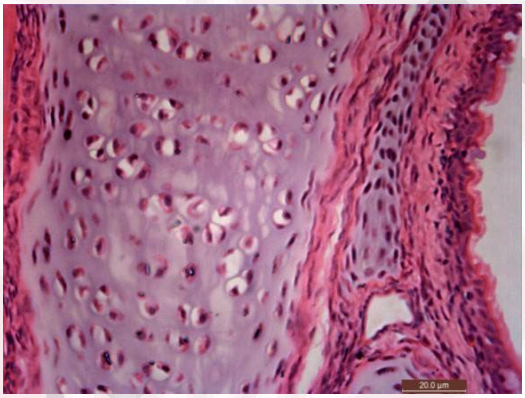

Figure 7: Trachea of chickens from group 6 showed normal to mild tracheitis with mild epithelial cells degeneration. HE. Bar= 20 µm

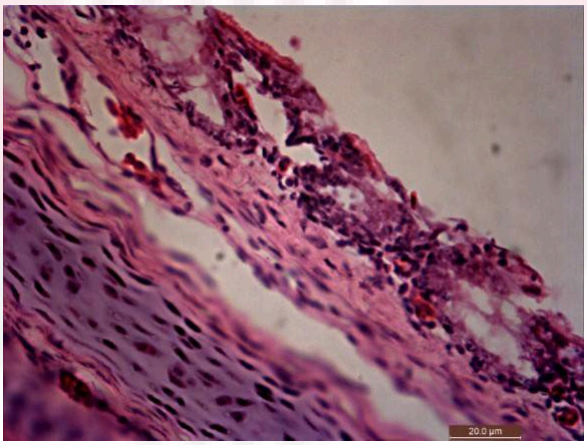

Figure 8: Trachea of the chickens from control group showing clinical signs had severe tracheitis with severe necrosis and degeneration of the epithelial cells. HE. Bar= 20 µm

5.0 Discussion

The study showed that the inoculated chickens either with VAG 5, VAG 6 and combination of VAG 5 and VAG 6 were 100 % protected compared to the chickens which were not inoculated based on the clinical signs, gross lesions and bacterial identification. Two out of four chickens from non-inoculated group showed clinical signs after challenged with VAG 6 via intramuscular route indicating only 50% of the chickens from the group were protected.

After the inoculation of inactivated APEC either with single VAG 5, VAG 6 or combination of VAG 5 and VAG 6, the inactivated APEC was able to stimulate a series of immune response which are the humoral and cell mediated response. According to Sadeyen *et al.* (2014), birds that were protected with inactivated APEC had significantly higher levels of serum IgY antibodies which was important for humoral immunity. Antibodies IgY contribute to defence against APEC with neutralization by causing the pathogens unable to attach to the cells, opsonisation where the pathogens will be coated with antibodies and they will be more readily to be phagocytised, and antibodies will also activate complement which facilitate the destruction of pathogens. According to Chaffer *et al*. (1997), APEC membrane vesicles can evoke both antibody production and T cell proliferation specifically stimulate cytotoxic T cells to confer resistance against APEC, further indicating inactivated APEC vaccines can stimulate both humoral and cell mediated immunity.

Besides, the inactivation method and adjuvant used can affect the efficacy of the inactivated bacterial vaccine and the outcome of immunization. According to Deb and Harry (1976), the chickens vaccinated with formalin-inactivated/alum-precipitated formula were resistant to intramuscular homologous challenge of *E. coli* up to 6 weeks when compared to bacteria inactivated with alcohol, heat or acetone. Adjuvant enhance the antigenicity of the bacterin and increase the recruitment and activation of antigen presenting cells (Leroux-Roels, 2010). Alum also function as delivery systems by generating depots that trap antigens at the injection site, providing slow release in order to continue the stimulation of the immune system so as to prolonged the immunity.

However, from this study, all the groups inoculated with inactivated APEC either with VAG 5, VAG 6, combination of VAG 5 and 6 or with booster were all 100% protected against VAG 6 challenge, for no mortality and clinical signs were observed from these groups of chickens. Therefore, differences among inactivated APEC with VAG 5, VAG 6, combination of VAG 5 and 6, or with booster in giving better protection against VAG 6 challenge was unable to be identified in this study. Since clinical conditions of APEC are difficult to be induced, body weight can be taken in the future to compare the performance of the broiler chickens of different groups as body weight and feed efficiency both decrease in broilers infected with APEC (Huff et al., 2006). Besides, ELISA can also be carried out to analyse the antibody titre of groups inoculated with inactivated APEC either with VAG 5, VAG 6, combination of VAG 5 and 6 or with boosters.

From this study, intramuscular route were able to cause infection in chickens compared to intranasal route because all the birds which were challenged via intranasal routes were 100% protected against the VAG 6 challenge. Intramuscular injection can bypass potential sites of host resistance such as mucosal defences of the intestinal or respiratory tract (Dziva, 2010). Hence, according to Deb and Harry (1976), intramuscular injection allowed correct assessment of the protective efficacy of inactivated vaccines. While for intranasal routes, though this routes closely resemble the natural route of infection, issues surrounding correlating inoculum dose and clinical disease need to be seriously considered as these are likely to affect reproducibility (Dziva, 2010). Colibacillosis has been known to be either opportunistic or secondary invaders, as they rarely occurred without a predisposing infection such as infectious bronchitis virus infection or an environmental stressful factor (Dziva, 2010). Besides, nasal, laryngeal and tracheal mucosal defences include mucus that trap bacteria and dust particles, which are propelled proximally by ciliary activity and subsequently coughed or swallowed. Hence, healthy tracheal mucosa is often difficult to be colonized by APEC (Dziva, 2010).

6.0 CONCLUSION

Inactivated APEC either with single VAG 5, VAG 6 or combination of VAG 5 and VAG 6, or booster could provide protection against APEC with VAG 6 infection compared to non-inoculated group.

7.0 RECOMMENDATION

Body weight and feed conversion ratio can be taken to evaluate the performance of the broiler chickens. The use of specific pathogen free chickens is more appropriate to evaluate the efficacy of vaccine as the immune status of commercial chicken is unknown and would interfere the results. ELISA can be used to evaluate the antibody titre among the chickens from different groups.

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