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The pathology of aorta of quails experimentally infected with *Enterococcus faecalis*

M.G. Al-hamdany and E.R. Al-kennany

Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

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Abstract

Isolation and identification of *Enterococcus faecalis* from small intestine and cecum of quails were done by culturing on differential and selective media. The lesions of aorta in quails experimentally infected with isolated bacteria were examined. Quails were divided randomly into four groups, the first group considered as control, and the other groups inoculated itraperitoneally with 0.5 ml of bacterial suspension of 10^8 CFU (2nd group), 10^9 CFU (the 3rd group), and 10^{10} CFU (the 4th group). The pathological changes of thoracic and abdominal aorta were recorded at 3, 7, 14 and 21 post infection days which include hyperplasia of endothelial cells with intensive localization of fatty vacuoles (foam cells) in intimal and medial layers of aortas with the proliferation and hypertrophy of vascular smooth muscle cells (VSMCs) accompanied by fragmentation of elastic fibers. These lesions were more severe at 14 and 21 post infection days. We concluded that *E. faecalis* possesses the ability to induce fatty degeneration in aorta indicating primary lesions of atherosclerosis in quails.

Keywords: Pathology; Aorta; Quails; *Enterococcus faecalis*
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دراسة مرضية للشريان الأبهر لطيور السمان المخمجة تجريبيا بجراثيم المكورات المعوية البرازية

محمد غسان الحمداني و انتصار رحيم الكناني

فرع الأمراض وأمراض الدواجن، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

الخلاصة

تم عزل وتصنيف جراثيم المكورات المعوية البرازية *Enterococcus faecalis* من الأمعاء الدقيقة والأعور لطيور السمان بعد زرعها وتنميتها على الأوساط الزرعية التفريقية والانتخابية ثم احدث الخمج التجريبي بهذه الجراثيم في السمان لملاحظة افات الشريان الأبهر. اشتملت الدراسة اربع مجاميع عشوائية من طيور السمان حيث اعتبرت المجموعة الأولى مجموعة سيطرة وحقنت المجاميع التالية 0.5 مل من المعلق الجرثومي داخل الخلب حيث حقنت المجموعة الثانية بتركيز 10^8 CFU وحقنت المجموعة الثالثة بتركيز 10^9 CFU أما المجموعة الرابعة فحقنت بتركيز 10^{10} CFU، تم ملاحظة الآفات المرضية للأبهر بعد إجراء الصفة التشريحية للسمان خلال الفترات ٣ و ٧ و ١٤ و ٢١ يوم بعد الخمج. تمثلت الآفات النسجية للأبهر بتضخم خلايا البطانة مع تموضع كثيف للفجوات الدهنية (خلايا رغوية) في طبقتي البطانة والمتوسطة مع تكاثر وضخامة خلايا العضلات الملساء الوعائية (VSMCs) رافقه تكسر الألياف المرنة وكانت هذه الافات اكثر شدة عند ١٤ و٢١ يوم بعد الخمج. نستنتج من هذه الدراسة أن جراثيم المكورات المعوية البرازية لها القدرة على احداث التنكس الدهني في الشريان الأبهر وهي مؤشر لحدوث الافات الأولية للتصلب العصيدي في طيور السمان.

Introduction

Japanese quails are to be found in all the continents. A number of lines, breeds and varieties have been developed for different production purposes. The biggest number of birds is to be found in South-East and East Asia, most often used for egg production (1). The prevailing breeds in Europe and USA are those of the combined and of the meat production type. The percentage content of edible meat in Japanese quail is very high (2).

Japanese quails are susceptible to both spontaneous and diet-induced atherosclerosis (3). It was investigated for their utility as a model for the discovery and evaluation of anti-atherosclerosis compounds. Although they possessed suitable characteristics for a screening animal, their development of atherosclerosis was too variable to make them a practical model. A search was conducted to find means to make quail uniformly atherosclerotic. To this end a line of quail susceptible to experimental atherosclerosis SEA were selectively bred. Thus, the (SEA) Japanese quail is a new animal model for atherosclerosis research (4).

During the last decades several studies have examined the potential role of oxidative stress in atherogenesis (5-7).

The oxidative stress hypothesis postulates that endogenous free radicals of unknown origin, possibly derived from mural cells, oxidize low density lipoproteins LDL and that oxidation products are allegedly responsible for initiation and progression of atherosclerosis (6).

In addition to production by host cells, bacteria can also produce superoxide anion. Production of superoxide by a clinical isolate of a *Streptococcus* D sp. strain was lytic for erythrocytes (8). Extracellular superoxide production has been reported to be a common trait in strains of *E. faecalis*. Among a total of 91 clinical and community isolates and type strains, 87 were found to produce detectable extracellular superoxide anion (9). Isolates associated with bacteremia or endocarditis produce significantly higher extracellular superoxide than those from the stool of healthy subjects (9,10).

For this when linking between susceptibility of quail to the atherosclerosis and ability of *E. faecalis* bacteria to produce reactive oxygen species ROS such as extracellular superoxide and hydrogen peroxide, this study occurred to know the ability of bacteria to induce aortic lesions.

Materials and methods

Eggs of Japanese quails (*Coturnix coturnix japonica*) were were obtained from Agricultural Research Corporation / Nineveh Research Department, incubated in the animal house in College of Veterinary Medicine - University of Mosul and the quail chicks were raised under standard laboratory conditions. The quails were used in the experiment when they became 21 days old and arrived to 80-100 gm weight.

E. faecalis isolated from the intestines and cecum of quail in sterilized method. The isolates cultured in brain heart infusion broth BHIB and incubated at 37 °C for 24 hours and then cultured on MacConkey agar and incubated at 37 °C for 24 hours. A swab was then taken and cultured on the differential media, Azide blood agar and Edward blood agar then incubated at 37 °C for 24 hours. Swabs from these media cultured on selective Enterococcus agar. The biochemical tests occurred by the API 20 Strep (company bioMérieux French) for the accurate diagnosis (11), were it incubated for 24 hours and read the result.

We obtain different concentrations of bacterial suspension of *E. faecalis* then randomly 80 quail birds aged 21 days divided into four groups, 20 birds for each group, the first group injected with normal saline as control group The second group injected with bacterial suspension 0.5 ml. intraperitoneally i.p of *E. faecalis* of concentration 10^8 CFU, third group injected with concentration of 10^9 CFU, the fourth group injected with concentration of 10^{10} CFU. The injected doses were.

Quails in each group were sacrificed at 3, 7, 14 and 21 days post infection. The samples were taken from thoracic and abdominal aorta and fixed in a neutral buffer formalin 10% and then embedded in paraffin, sectioned at 5 µM, and stained with hematoxylin and eosin (H&E). Selected sections were stained with Gram's tissue modified stain for bacteria, Alcian blue stain (AB) pH-2.5 for acidic mucopolysaccharides such as glycosaminoglycans in aorta, and Masson's trichrome stain and Van Gieson stain for collagen fibers (12).

Results

Enterococci bacteria grew on the Azide blood agar as white to gray color colonies, white pinhead size colonies on the Edward blood agar and single pinhead size red to brown color colonies on the selective Enterococcus agar.

The biochemical test API 20 Strep that showed that the type of bacterial isolation is *E .faecalis* 99% as calculates the final number and compares with the guide of bacteria.

The histopathological changes at 3 days post infection (p.i) were hyperplasia of endothelial cell with intensive localization of fatty vacuoles in intimal layer and little in the medial layer with the proliferation and hypertrophy of vascular smooth muscle cells (VSMCs) began toward intimal layer accompanied by fragmentation of elastic fibers (Figure 1). At 7 and 14 days p.i. the lesions were more extensive proliferation and hypertrophy of endothelial cells, beginning of formation of recent thrombus attachment to endothelium, very extensive localization of fatty vacuoles (foam cells) in medial and intimal layers, fragmentation of elastic fibers and degeneration of VSMCs (Figure 2), while at day 21 p.i the foam cells were in all layers of the aorta, fragmentation of elastic fibers with the hyperplasia and proliferation of VSMCs associated with infiltration of mononuclear inflammatory cells in 3rd group inoculated with 10^9 CFU of bacterial suspension (Figure 3).

Alcian blue (pH-2.5) stain has revealed an accumulation of acidic mucopolysaccharides between the disrupted elastic lamellae of the media and proliferation and hypertrophy of VSMCs toward intimal layer, and presence of bacterial colonies in the intima at 3 days p.i (Figure 4),

and severe positive reaction of blue mucinous ground substance in the medial at 14 days p.i (Figure 5).

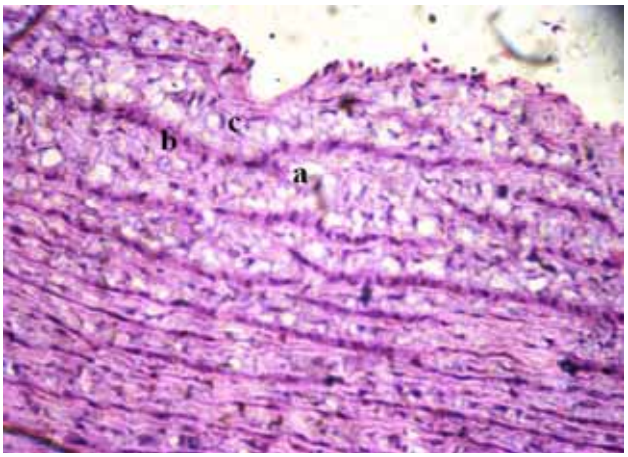

Figure 1: Quail's aorta, 3 days p.i showing deposition of fat vacuoles in intima and media layers (a) accompanied by proliferation and hypertrophy of VSMCs toward intimal layer (b) and fragmentation of elastic fibers (c). H&E stain X350.

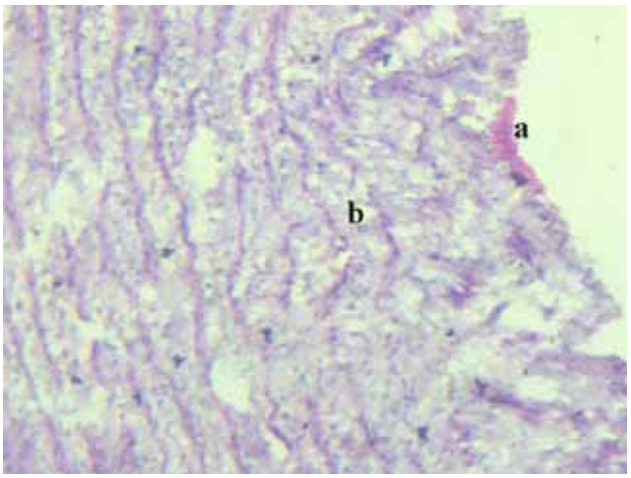

Figure 2: Quail's aorta, 14 days p.i showing formation of recent thrombus attachment to endothelium (a) and very extensive localization of foam cells in all layers of the aorta (b), in 4th group inoculated with 1010 CFU bacterial suspension. H&E stain X350.

Masson's trichrome stain demonstrated collagen rich extracellular matrix in the media of the aortic wall with hyperplasia and proliferation of VSMCs at 7 days p.i in the 4^{th} group inoculated with 10^{10} CFU of bacterial suspension (Figure 6) and at 21 days p.i, there were severe hyperplasia and proliferation of VSMCs and lysis of collagen fibers (Figure 7). Van Gieson stain showed fragmentation of elastic fibers with hyperplasia and proliferation of endothelial cell and presence of bacterial colonies in the intima at 7 days p.i (group inoculated with 10^9 CFU of bacterial suspension) (Figure 8).

These aortic lesions were in the all bacterial concentrations but much more in the 10^9 CFU and 10^{10} CFU compared with control.

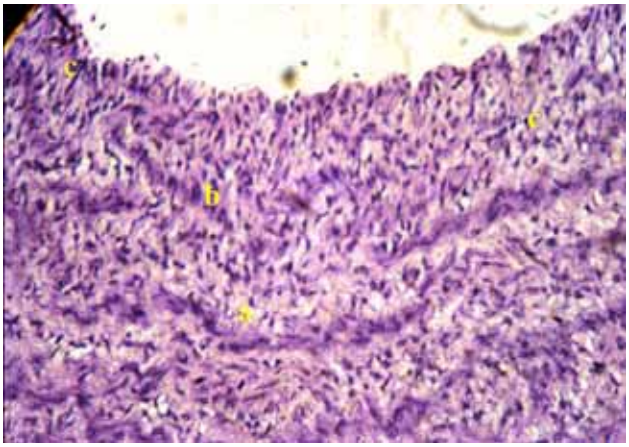

Figure 3: Quail's aorta, 21 days p.i, showing foam cell in all layers of the aorta (a), fragmentation of elastic fibers with the hyperplasia and proliferation of VSMCs (b) associated with infiltration of mononuclear inflammatory cells in 3rd group inoculated with 109 CFU bacterial suspension (c). H&E stain X350.

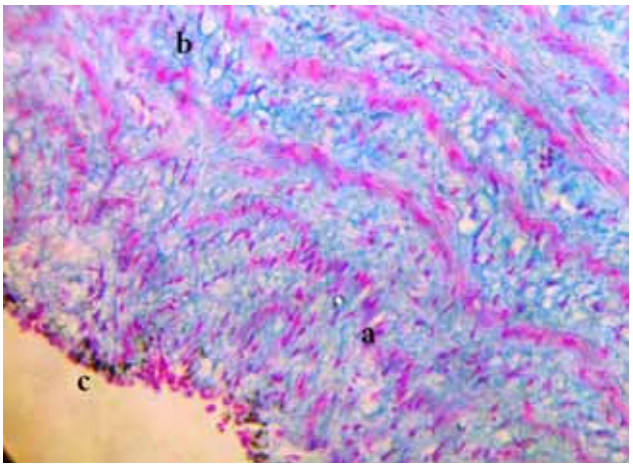

Figure 4: Quail's aorta, 3 days p.i, showing proliferation and hypertrophy of VSMCs toward intimal layer (a) accompanied by acidic mucopolysaccharides between the disrupted elastic fibers (b) and presence of bacterial colonies in the intima in black color (c), Alcian blue (AB) pH-2.5 +ve. X350.

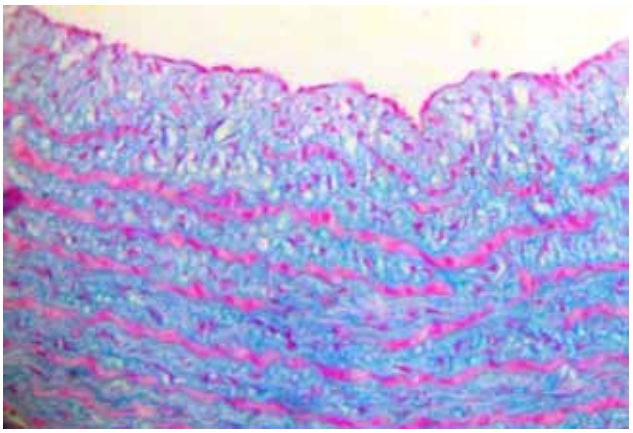

Figure 5: Quail's aorta, 14 days p.i, showing severe positive reaction of blue mucinous ground substance in the media. Alcian blue (AB) pH-2.5 ++ve X350.

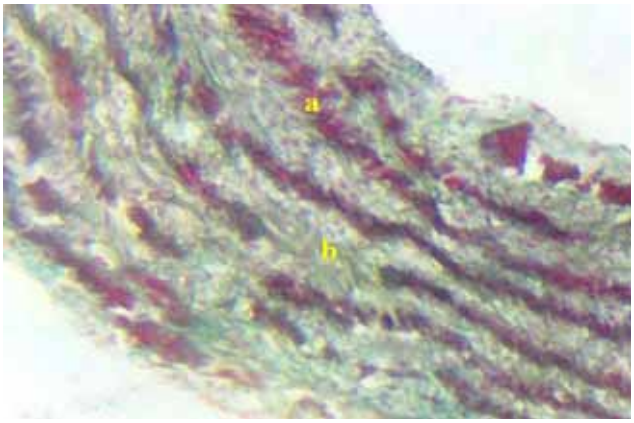

Figure 6: Quail's aorta, 7 days p.i showed hyperplasia and proliferation of VSMCs (a) and disrupted collagen rich extracellular matrix in the media of the aortic wall (b) in 4th group inoculated with 1010 CFU bacterial suspension. Masson's trichrome stain X270.

Discussion

Cultural, staining and biochemical features of the isolated bacteria indicated that it was *E. faecalis*, this is consistent with other researchers (13-15). The choice of three concentrations of bacterial suspensions (10^8 CFU, 10^9 CFU and 10^{10} CFU) was based on other studies of experimental bacterial infection of *E. faecalis* where it was stated (16) that the LD_{50} for these bacteria in mice reach to 3.2 x 10^8 CFU - 2.2 x 10^8 CFU by inoculation i.p and cause death after 24-36 hours. However, in this study a preliminary trial showed that these concentrations did not cause mortality, and therefor they were used in this study.

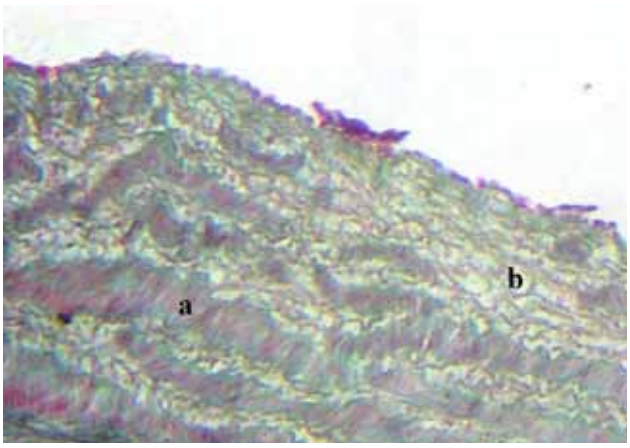

Figure 7: Quail's aorta, 21 days p.i, showed hyperplasia and proliferation of VSMCs (a) and disruption of collagen fibers (b). Masson's trichrome stain X350.

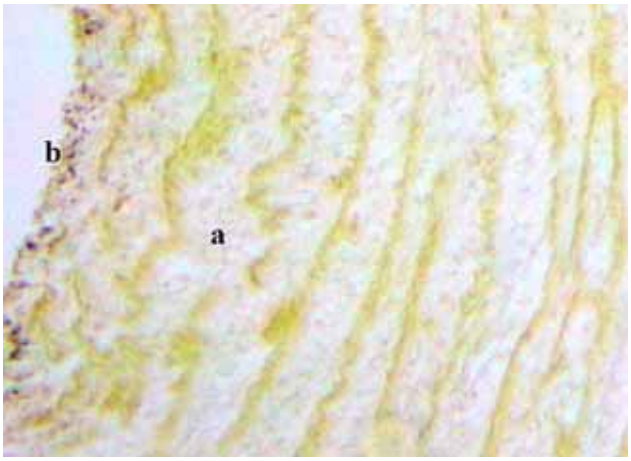

Figure 8: Quail's aorta, 14 days p.i, showing fragmentation of elastic fibers (a) with hyperplasia and proliferation of endothelial cell and present of bacterial colonies in the intima in black-brown color (b). Van gieson stain X350.

The clinical signs included restlessness, reflect feathers, depression that were similar but less severe to that occurred in chicken infected experimentally by *E.faecalis* (17). The pathological changes at 3, 7, 14 and 21 days p.i. showed appearance of it at 3 days p.i. that may be occurred due to bacteremia which has been confirmed by re-isolation of *E. faecalis*. The appearance of lesions may be happened due to the virulence factors of *E. faecalis*, which may relate to colonization of the host, competition with other bacteria, resistance against defense mechanisms of the host, and production of lesions directly through production of toxins or indirectly through induction of inflammation. The factors most extensively studied are: aggregation substance,

surface adhesins, sex pheromones, lipoteichoic acid, extracellular superoxide, gelatinase, hyaluronidase, and cytolysin (hemolysin) (18).

As the *E. faecalis* bacteria have the ability to produce reactive oxygen species ROS such as extracellular superoxide and hydrogen peroxide H_2O_2 which are virulence factors that cause oxidative damage that lead to disruption of lipids of cell membrane by lipid peroxidation and thus freeing fat (19), so the idea for this study came to see aortic lesions caused by *E.faecalis* in quail.

The appearance of aortic lesions in thoracic and abdominal aorta in quail confirms the sensitivity of these animals to occurrence of atherosclerosis (20,21) which were indication to occurrence of primary lesions of atherosclerosis in agreement with (7,22) in chicken and rats, as well as the ability of *E. faecalis* bacteria to cause oxidative damage and atherosclerotic-like lesions (22-24), and it may be happens through the transmission of bacteria in the blood vessels through the blood after being inoculated i.p. This bacteria owning many virulence factors aforementioned which able the bacteria to stick on platelets and then to the endothelial cells of aorta. This may explain the hypertrophy and hyperplasia of the endothelial cells as a response to adhesion of these bacteria, but the process of formation of fat vacuoles as the fat accumulation in cytoplasm of VSMC and monocytes, which called foam cells in the two layers of intima and media of the vessel wall as a result of peroxidation and localization of low density lipoprotein LDL-C by the reactive oxygen species ROS produced by *E. faecalis* or through the leukocytes as inflammatory response to it (25), where is the ability of monocytes to peroxidation of LDL-C occurs by the presence of attracted receptors on its surface thereby forming foam cells and to stimulate the VSMC to liberalization the proteins outside the cell, which include proteoglycans and collagen and elastic fibers which involved in formation of fat droplets (25,26). Foam cells characterize the atherosclerotic lesions and it is believed that they are derived from hematogenous macrophage and VSMC (27).

The mechanisms that cause localization of monocytes and T cells in the arterial wall demonstrated by endothelial cells of leukocyte adhesion molecules and on chemotactic proteins and factors that facilitate the recruiting, expanding, and sustaining of the monocyte/macrophage population (28). Proliferation of aortic or VSMC, an important feature of primary atherosclerosis, occurs in the early stage of the disease in Japanese quail (29). Recent studies showed that composition of the ECM such as proteoglycans PGs link to LDL with high affinity, which is a basic precondition for the accumulation of lipid droplets in the intima (30,31). A large amount of PGs in the subendothelium of aorta in the fatty vacuoles stage, which is much higher than in normal subendothelium and subendothelium of the initial phase, develops as a result of the synthetic activities of proliferating VSMCs. Although in the unmodified subendothelium aorta, as well as at the initial stage of atherosclerosis, there is a certain amount of acidic PGs, it has been proven that the PGs synthesized by proliferating cells contains 30% more acidic sulfated PGs, some of which are atypical of normal vascular tissue (32). In the composition of newly synthesized PGs, heparan sulfate, versican, biglycan and keratan sulfate proteoglycan are predominantly represented (32,33), so in sections in which the acid mucopolysaccharides (AMP) were stained with Alcian blue (AB) pH 2.5, an increase of Alcian blue-positive substance was noted highly sulphated mucins (proteoglycans),which are a characteristic of the early stage of lesion.

The various components of connective tissue, such as collagen, elastic fibers, and ground substance, and cellular elements, as well, had undergone disruption. Masson trichrome stain may reflect a greater contribution of decreased collagen fibers than an absolute increase in the lipids plaque, which indicate that cellular lipid metabolism including an increase in cholesterol efflux that decreases extracellular matrix ECM content (34). In addition to collagen, we examined the vascular ECM protein elastin by Van Gieson stain, we found increased disruption of elastin lamellae as primary atherosclerosis progressed, as previously reported (35,36). Elastin breaks were defined as interruptions in the elastin fiber, expressed as number of elastin breaks per square millimeter of medial area (37).

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