Clinicopathological and Hematological Changes in Consequence to Experimental Infection of Rabbits with Pasteurella Multocida Type A

Abeer HM El-Hendy¹, Fatma M Mohamed²*, Ahmed K Hassan³

¹Pathology Department, Assiut Regional Laboratory, Animal Health Research Institute, Agricultural Research Center (ARC), Egypt, ²Poultry Diseases, Assiut Regional Laboratory, Animal Health Research Institute, Agricultural Research Center (ARC), Egypt, ³Department of Poultry diseases, Faculty of Veterinary Medicine, Assiut University, Egypt

Abstract

The current research aimed to study clinical, hematological and pathological changes occurred due to experimentally challenged rabbits with Pasteurella multocida type A. Twelve healthy Pasteurella multocida free rabbits were divided into control and challenged groups. Rabbits of the challenged group were intra-nasally inoculated with 0.5 ml of 1x10⁵ CFU of Pasteurella multocida type A, while rabbits of the control group were inoculated with 0.5 ml of brain heart infusion broth (BHI). Clinical signs, hematological parameters and histopathological lesions were recorded for 14 days. Rabbits of challenged group showed nasal rubbing with minimal mucus discharge, conjunctivitis and reduction in spontaneous activity, but no mortality occurred. Hematologically, there was highly significant low RBCs count with concomitant significantly reduction of PCV in challenged rabbits, but hemoglobin concentration was insignificantly low in challenged animals as compared to control. Although, there was insignificant increase of WBCs count in challenged group, neutrophils and monocytes recorded significant and highly significant high value in challenged rabbits than control, respectively. Challenged rabbits, however demonstrated highly significant lymphopenia, but other blood parameters did not show any significant differences between challenged and control rabbits. Histopathologically, challenged animals showed meningitis, congestion and degenerative changes in respiratory epithelium, bronchopneumonia, vacuolar degeneration of hepatocytes and vascular changes in heart and spleen. Conclusively, the current study verified that challenging rabbits with Pasteurella multocida serotype A was not lethal and although it was essentially pneumotropic in nature, it caused lesions in other visceral organs, which in absence of bacteremia, could be caused by bacterial endotoxin liberated during disease pathogenesis.

Keywords: Experimental infection, Hematological parameters, Histopathological lesions, Pasteurella multocida.

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*Corresponding Author: Fatma M. Mohamed E-mail: elzuhry@yahoo.com

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Introduction

Rabbit pasteurellosis considered one of most significant causes of economic losses in large production units throughout the world (Stelian et al., 2011). It is bacterial disease caused by Pasteurella multocida, which is considered a normal opportunistic microorganism of upper respiratory tract in a range of animal species (Suelam and Samie, 2011) including cattle, swine, canine, fowl and rabbits (Harper et al., 2006; Hotchkiss et al., 2011, Suelam and Samie, 2011). Pasteurella multocida is a Gram-negative coccobacillus, non-motile, non-spore-forming and facultative anaerobic bacterium that shows bipolar staining with Giemsa stain (Carter, 1987).

By using serological techniques P. multocida is classified on the base of capsular antigens into five capsular serogroups (A, B, D, E and F), and into 16 somatic serotypes (1-16), based on lipopolysaccharide antigens (Rimler and Rhoades, 1989). Rabbit pasteurellosis mainly caused by the capsular type A and a lesser extent by capsular type D strains (Dabo et al., 1999; Katoch et al., 2015), that may vary in clinical symptoms as (respiratory distress, conjunctivitis, otitis, genital infection, meningitis and abscesses) also P. multocida may don’t show any clinical signs (Delong and Manning, 1994; Asran et al., 2016). The clinical signs variability as well as the course of the disease may be influenced by different virulence factors of P. multocida such as polysaccharide capsule, lipopolysaccharides (endotoxins), fimbria, exotoxins, extracellular enzymes and plasmids (Harper et al., 2006) .The infection often acquired from a carrier dam and the disease developed when the animals are exposed to stress factors like transportation (Asran et al., 2016). It has been estimated that the prevalence of P. multocida in clinically healthy animals (rabbits or other animal species) range from 20 to 90% depending on the detection methods applied (Sanchez et al., 2004), and it commonly affect rabbits at age of 4 to 8 weeks but rabbits older than 8 months to one year exhibited lesser incidence (El-Ghawy, 1972). It was reported that intra-nasal inoculation of rabbit with P. multocida resulted into marked inflammation in respiratory tract leading to congestion, hemorrhages, edema, and infiltration of inflammatory cells with fibrin deposition resulting in to fibrino-suppurative bronchopneumonia (Patel et al., 2016). It was estimated that P. multocida infection in rabbits cause significant changes only in erythrocytes, hemoglobin, hematocrit and mean concentration of corpuscular hemoglobin (Petrova et al., 2017). This research work aimed to study the clinical, hematological and histopathological changes in rabbits experimentally infected with P. multocida type A.

Materials and Methods

Animals

The study was conducted on 12 healthy pasteurellosis free, 11 to 12 weeks age adult New Zealand White (NZW) rabbits of either sex (weighing 1000-1200g and aging 11-12 weeks) obtained from Rabbit Production Unit, Faculty of Agriculture, Assiut University, Assiut, Egypt. In addition, two adult male mice were obtained from the Animal house, Faculty of Veterinary Medicine, Assiut University, Egypt. Rabbits and the two mice were maintained under standard management conditions. For feeding, conventional standard laboratory diet was used with free access to water. Animal care, housing, and environmental conditions (temperature, humidity and light dark cycle) were applied according to the recommendations in the
Guide for Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) before and during the study period. Animals were identified by the use of permanent marker on the ear.

Pasteurella multocida live culture:

*P. multocida* live culture (of rabbit origin) was obtained on 5% blood agar slant from Serology Unit and Bacterial Strains Bank, Animal Health Research Institute, Dokki. The isolate was streaked onto different blood agar plates to obtain an isolated colony for further use in experiment.

Passage in mice for virulence:

A loopful of the culture was dissolved in 0.5ml sterile PBS and injected in mice intraperitoneally using an insulin syringe. Following the death of mice after 48h, postmortem was done under aseptic conditions. Blood was collected from heart and streaked on 5% blood agar, incubated overnight at 37oC. Separate colonies were obtained and used for inoculation in healthy rabbits.

Pre-inoculation testing:

The experimental rabbits were checked for *P. multocida* free status. Sterile swab was deeply inserted in either nares of rabbits and cultured on 5% blood agar media. The grown culture on blood agar was subjected to Giemsa staining. The smears were free from the bipolar *P. multocida coccobacilli*.

Experimental design

Twelve healthy *P. multocida*-free-rabbits were divided into two groups (control and challenged), six animals each. 0.5 mL of 1x105 CFU of prepared inoculum was inoculated in each nares using an insulin syringe without needle. Just after inoculation, the head of rabbits were kept in an upward position for 2 min to ensure that the inoculum moved deeply in nasal cavity. The rabbits of the control group were inoculated with 0.5 ml uncultured BHI broth in each naris in the same procedure. Animals were observed for 14 days post-inoculation for clinical signs and mortalities. Body temperature was recorded daily with digital thermometer through the rectum.

Sample collection, gross necropsy and histopathology:

Before necropsy, blood was collected from ear vein in sterile vials with anticoagulant for hematology. At the end of the experiment, animals were sacrificed and necropsied. Samples from the trachea, liver, spleen, heart, kidney and brain were collected in 10% neutral buffered formalin (NBF) for histopathology. Before fixation, impression smears were prepared from the above-mentioned organs, fixed in methanol and stained with Methylene Blue (EMB) stain for examination of bipolar organisms. Fixed tissues were processed routinely for histopathology according to Bancroft and Gambl (2008). Thin paraffin sections (3-5µm) of the tissues were stained with Hematoxylin and Eosin (H & E) (Suvarna et al. 2013), examined microscopically and photographed.

Hematological indices:

At the end of experiment (14th day) blood samples were collected from ear veins from each animal. About 2ml of blood were collected into sterile (EDTA) anticoagulant containing vials for determination of the following hematological parameters; red blood cell (RBC) count, hemoglobin (Hb)
concentration, packed cell volume (PCV), mean corpuscle volume (MCV), mean corpuscle hemoglobin concentration, white blood cell (WBC) count and differential leukocytic count using (Medonic Auto Hematology Analyzer CA620/Vet/20)

Statistical analysis:
Data of hematological parameters are expressed as mean ± SD. The statistical analysis was performed by using SPSS data analysis software (version 17). The analysis was performed by one-way ANOVA followed by Scheffe and Duncan test, P value < 0.05 is considered as significance.

Results
Clinical observation
The rabbits of the control group revealed no clinical signs, no mortality and no postmortem lesions among them. After 24 hours of intranasal inoculation with P. multocida, the rabbits of challenged group demonstrated nasal rubbing with minimal mucus discharge and reduction in spontaneous activity. Symptoms also included sneezing, affected rabbits made a loud snoring sound or snuffling. Three of the challenged rabbits were demonstrated grooming their faces and nasal discharge appeared on their front paws. Two rabbits demonstrated conjunctivitis and crustings around the eyes. None of these challenged animals died during the two weeks of observation.

Hematological parameters
The hematology of challenged rabbits revealed striking variations in different parameters as compared to control animals. RBCs count demonstrated highly significant reduction; as a consequence hemoglobin is insignificantly low in challenged rabbits as compared to control. The WBCs revealed non-significantly high values in challenged group as compared to control. In addition, PCV demonstrated significantly low values in challenged group as compared to control. There was non-significant difference between challenged and control animals in MCV. The MCHC, however, demonstrated significantly high value in challenged when compared with control group. Concerning the differential leukocytic count, neutrophils and monocytes recorded significant and highly significant high value in challenged rabbits than those of the control, respectively. Lymphocytes, however, demonstrated highly significant low values in challenged as compared to control animals. No significant difference was demonstrated in the eosinophils (Table 1 and Fig. 1).

Table 1: shows the detailed changes in different hematological parameters (Means ± SE) in rabbits of control and challenged groups. *Significant (p<0.05), **Highly significant (p<0.01)

| Parameters   | Control group | Challenged group |
|--------------|---------------|------------------|
| RBCs (10⁶/mm³) | 5.62±0.12     | 4.70±0.23**      |
| Hb (g/dl)    | 10.39±0.34    | 10.07±0.71       |
| PCV (%)      | 37.95±0.45    | 34.09±1.88*      |
| MCV(FL)      | 71.64±1.23    | 69.49±1.48       |
| MCHC (g/dl)  | 30.73±0.48    | 34.10±0.63*      |
| WBC (10³/mm³)| 7.21±0.20     | 7.56±0.33        |
| Neutrophil (10³/mm³) | 2.60±0.20 | 3.56±0.21*        |
| Eosinophil (10³/mm³) | 0.12±0.007    | 0.14±0.01       |
| Monocyte (10³/mm³) | 0.26±0.01 | 0.89±0.04**       |
| Lymphocyte (10³/mm³) | 4.30±0.22 | 2.90±0.20**        |
Pathological observation:

The challenged animals demonstrated various histopathological lesions in the examined organs.

Brain

Challenged animal showed congested mildly thickened meninges as revealed by gross examination. Microscopically, two rabbits showed some evidence of meningitis in form of lymphocytic inflammatory cells infiltration, hyperemia of blood vessel and edema (Fig. 1 A, B). The pathological changes in the brain glial reaction associated with neuronal degeneration (Fig. 1 C, D).

Fig. 1. Brain of rabbit 14th d post infection with Pasteurella multocida showing (A) Lymphocytic inflammatory cells infiltration in meninges; (B) Infiltration of inflammatory cells, hyperemia of blood vessels and edema (arrows); (C) and (D) Brain showing edema surrounding blood vessels, glial reaction associated with neuronal degeneration (arrows) (H&E x400).

Trachea

At time of necropsy tracheal mucosa was moderately reddened and edematous. Microscopically, there was increased number of goblet cells in the tracheal mucosa and submucosal congestion (Fig. 2A). In some parts, the lining epithelium showed desquamation, polymorphonuclear neutrophils, lymphocytes and macrophages infiltrated among the degenerated epithelial cells and in the sub epithelial tissue (Fig. 2B).

Lung

Grossly, there was mild congestion and variable degrees of pneumonia in the anterior lung lobes with emphysematous areas. Microscopically, the lung showed thickening of inter alveolar septa, mononuclear cellular infiltration, hyperplastic bronchiolar epithelium and fibrinoid necrosis in the wall of pulmonary blood vessel (Fig. 2 C, D). In addition, scattered hemorrhages were demonstrated in
the alveolar lumen and alveolar emphysema (Fig. 2 D-F).

**Fig. 2.** Trachea and lung of rabbit 14th d post infection with *Pasteurella multocida* (A) Trachea shows hypertrophy of goblet cells (arrows), congested and dilated blood vessels (B) Desquamation of epithelial lining (arrow), neutrophils and macrophages infiltration between the degenerated cells and in the sub-epithelial layer and edema in lamina propria (arrow head) (H&Ex400) (C) Lung shows thickening of inter-alveolar septa (D) Congested pulmonary blood vessels, peri bronchial and peri vascular inflammatory cells infiltration (arrows) (H&Ex100). (E) Intra-alveolar scattered hemorrhages (arrows) (F) Thickening of inter-alveolar septa and emphysema (H&Ex400).
Liver

At necropsy, there were no appreciable lesions in the liver of infected rabbits except congestion. Microscopically, there was diffuse hydropic degeneration and cytoplasmic vacuolation of hepatocytes, marked congestion of blood vessels (Fig. 3A), scattered necrotic foci infiltrated with mononuclear inflammatory cells (Fig. 3B). In addition, there was periportal mononuclear inflammatory cells infiltration associated with edema and fibrosis (Fig. 3 C, D).

Heart

At the time of necropsy mild congestion could be observed in the heart but spleen did not show any gross changes. Microscopically, the lesions in the heart consisted of hemorrhages, engorged blood vessels (Fig. 4 A, B).

Spleen

In the spleen the most observable lesion was slight increase of hemosiderin laden macrophages in red pulp, mild congestion and hemorrhages (Fig. 4C, D).
Fig. 4. Heart and spleen of rabbit 14th d post infection with *Pasteurella multocida* (A) Heart shows interstitial hemorrhage (arrows). (B) Congested blood vessels (arrows) (H&Ex400). (C) Spleen shows congestion and hemorrhages and slight increase in hemosiderin laden macrophages (arrows) (H&Ex100). (D) Higher magnification showing hemosiderin-laden macrophages in the red bulb of the spleen (arrows) (H&Ex400).

**Discussion**

The current study aimed to study pasteurellosis in following intranasal challenge with *P. multocida* serotype A. In agreement with Mir et al. (2001) and Praveena et al. (2010), the symptoms which were observed within 14 days after challenge, in the current investigation, started with acute form then subsided to subacute form of the disease. Signs included primarily dullness, rhinitis, sneezing, bronchopneumonia and conjunctivitis, but then decline to subacute form with minimal clinical signs. However, no deaths were observed during the whole experiment. As previously mentioned, *P. multocida* isolated from rabbits demonstrate great differences in the virulence ranging from chronic form of rabbit pasteurellosis (snuffles) to fatal septicemic forms (Glavits and Magyar, 1990). It has been also stated that isolates of *P. multocida* vary in their abilities to induce disease; some are primarily associated with inflammatory conditions in the upper respiratory system, while others may cause septicemia and pneumonia (Chengappa et al., 1982; DiGiacomo et al., 1991). In addition, some other investigations reported
dyspnoea and abdominal breathing in addition to the above-mentioned symptoms (Al-Haddawi et al. 2001, Jaglic et al. 2006, Ramesh Kumar et al. 2006).

Regarding hematological parameters, the rabbits of challenged group revealed striking variations as compared to control animals. Although RBCs demonstrated highly significant low count with concomitant significantly reduction of PCV in challenged rabbits, hemoglobin concentration was insignificantly low as compared to control. Similarly, Nassar et al. (2013) and Alam et al. (2018) reported that there was significant reduction in RBC count and PCV% in P. multocida infected rabbits. The low RBCs count, PCV and hemoglobin accompanying P. multocida infections may be due to decrease in the iron release from macrophages to plasma cells with subsequent decrease in RBCs life span (Hoffbrand and Pettit, 1993). In our opinion, the reduction in the RBCs count, PCV and hemoglobin concentration could be attributed to the lysis of RBCs as a result of bacterial toxin accompanying P. multocida infections.

In spite of the insignificant increase of WBCs count in challenged group, neutrophils and monocytes recorded significant and highly significant high value in challenged rabbits than those of the control, respectively. However, Al-Jeboori and Rasheed (2014) demonstrated marked leukocytosis associated with increase percentages of the neutrophils, monocytes and lymphocytes in infected group in comparison to control. Contrarily, the current study showed that lymphocytes demonstrated highly significant low values in challenged as compared to control animals. Leukocytosis (increased count of WBCs in blood) has been stated to occur as a result of activation of bone marrow by P. multocida antigens (Numata et al., 1998; Carrigan et al., 2004). The increase in the percentages of neutrophils, monocytes, has been attributed to the bacterial toxin of P. multocida (Hoffbrand and Pettit, 1993). In the same concern, Ruble et al. (1999) and Alam et al. (2018) believed that neutrophilia in blood of P. multocida infected rabbits could be a physiological response from the body to minimize the spread of P. multocida infection. While the lymphopenia observed in rabbits of challenged group in our study could be possibly due to infiltration of these cells into the infected tissues or as a result of apoptosis or cytolysis by bacterial toxins (Praveena et al., 2010).

The histopathological changes observed in brain included meningitis, vascular edema and neuronal degeneration. Similarly, Kpodekon (1983) found that rabbits experimentally infected with P. multocida through infraorbital nerve, oral rout and intravenous rout revealed meningitis, encephalitis and/or otitis. Kpodekon (1983) assumed that this nerve lesion triggered stagnation of neuronal lymph which provoked retrograde centripetal circulation of lymph up to the brain resulting in meningitis. Also, Patel et al. (2016) recorded meningitis in three rabbits experimentally infected with P. multocida B2 through intranasal rout and observed bacterial emboli in the blood vessels of the brain. Contrarily, P. multocida meningitis has been reported to be rarely occurring in humans (Green et al., 2002).
challenged rabbits in the present study were consistent with that previously finding of Mir et al. (2001), Katoch et al. (2015) and Patel et al. (2016). Tracheal lesions, in the current investigation, were characterized by goblet cells hyperplasia and hypotrophy as well as neutrophils infiltration associated with desquamation of degenerating epithelial cells. The degeneration of epithelium probably, attributed to the effect of inflammatory cells when they migrate through the epithelium. Similarly, an increase in the number of goblet cells in the respiratory epithelium in rabbit fetuses exposed experimentally to P. multocida infection has been reported previously (Carrillo et al., 2012). Cells contain small secretory granules and are thought to produce watery secretions. Goblet cells have been reported to originate from other cell type in the respiratory epithelium (club cells), which act as progenitors of goblet cells after bronchiolar injury (Sleigh et al., 1988; Zhu et al., 2008). The increase in the goblet cell population in the respiratory epithelium following P. multocida infection in rabbits is a logic reaction because goblet cells are involved in mucin production that has defense mechanism against infections particularly in respiratory tract (Shuter et al., 1996; Scharfman et al., 1996). The prominent vascular lesions detected in lungs and trachea were consistent with observation of (Katoch et al., 2015; Patel et al., 2016; Alam et al., 2018). Our findings support those of Praveena et al. (2010) who verified the important role of pulmonary intravascular macrophages in induction of lung lesions in pasteurellosis, they mentioned that bacterial endotoxin activate these macrophages and may lead to influx of other inflammatory cells and cascading injury. They added that toxins products of P. multocida alone or in combination with products of inflammatory cells induce necrotic changes in the wall of pulmonary blood vessels and the injured wall of blood vessels may be responsible for edema and hemorrhages in alveoli. Additionally, Patel et al. (2016) reported that the bacterial toxin also causes necrosis of leukocytes with production of oat cells which become marked by streaming pattern of condensed chromatin material.

Although previous reports verified that pneumonic pasteurellosis is the main manifestation of the disease in rabbits challenged intranasally with the P. multocida (Jaglic et al., 2008), other parenchymatous organs like liver were also affected. The histopathological changes observed in the liver of challenged rabbits in the current study included diffuse hydropic degeneration of hepatocytes, few necrotic foci infiltrated with inflammatory cells, periportal mononuclear inflammatory cells infiltration associated with edema, congestion and fibrosis. These findings are consistent with previous results in experimentally infected rabbits with P. multocida (Alam et al., 2018), and P. multocida experimentally infected mice as well (Praveena et al., 2010). The most significant microscopic lesions observed in heart and spleen of challenged rabbits those reported by (Praveena et al., 2010) in P. multocida infected mice. These lesions included an acute cell injury that may be resulting from bacterial endotoxins or toxic proteins (Praveena et al., 2010).

Conclusion

It could be concluded from the current study that infection with P. multocida
serotype A was not lethal to rabbits. Although the strain was essentially pneumotropic in nature, yet it caused lesions in other visceral organs, which in absence of bacteremia, could be attributed to the bacterial endotoxin during the pathogenesis of the disease.

**Conflict of interest statement**

The authors declare that they have no conflict of interest.

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