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Demonstration of Pigeon Circovirus Infection in Western-Libya, Based on Histopathology, Electron Microscopy and Apoptosis

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Abstract: Pigeon circovirus (PiCV) infection associated with high morbidity and mortality have been recognized in a large rearing loft for racing pigeons in El-Nekat El-Khams-Western Libya. There exist a number of data indicating that immunosuppression with subsequent infections with various secondary microbial infections are a consequence of PiCV infection. PiCV diagnosis was based on light and transmission electron microscopical observation of characteristic botryoid crystalline globular inclusions of circovirus in bursa of Fabricius as well as the potential concurrent lesions of other primary and secondary lymphoid tissues. Histopathological findings were suggestive of primary bursotropism of pigeon circovirus, followed by secondary systemic spread from the bursa of Fabricius, particularly to non-bursal lymphoid organs. The last stage of the disease was associated with various secondary (particularly bacterial) infections. *In situ* detection of apoptosis in the bursa of Fabricius indicated that PiCV was concomitant with an increase in bursal lymphocytic apoptotic events related to viral infection and leading to severe acquired immunosuppression. It was concluded that the present trial has brought an ultimate proof of the presence of pigeon circovirus in Western-Libya and further investigations and a method based on the presence of viral DNA using *in situ* Hyperdization or nested PCR are needed to evaluate the prevalence of PiCV.

Key words: Pigeon circovirus · Prevalence in libya · Pathology · Apoptosis · Electron microscopy

INTRODUCTION

Pigeon Circovirus infection (PiCV) is a contagious disease affecting primarily young pigeons [1, 2]. Mortality is variable, but it can approach 100%. Clinical signs and mortality are primarily due to an apparent virus-induced immunosuppression with subsequent infection with various secondary viral, bacterial, fungal and parasitic agents. It is an emerging disease which was first reported in California in 1993 [3] and have recently described in an increasing number of avian species [4]. In fact, clinical signs are often associated with the secondary infections with agents such as Paramyxovirus-1, Poxvirus, adenovirus and herpsvirus [3].

Circovirus -like particles were seen in negatively stained preparations of faeces from pigeons in South Africa during the same year [5]. However, suspicious Circovirus-like inclusions were noted prior to this [6]. Circovirus was reported in racing pigeons from Northern Ireland and England during 1995 and 1996, respectively [1, 2]. The later reports suggest that PiCV is wide spread in European pigeons prior to its spread to the Middle East.

Three other current circoviruses members of the Circoviridae family are known to be causal agents of spontaneous diseases: chicken anemia virus (CAV) infection in fowl, psittacine beak and feather disease (PBFD) virus in psittacine birds and porcine circovirus that causes post-weaning multisystemic wasting syndrome in pigs [7,8].

These viruses share many epizootiological and pathological similarities (i.e. young age of affected animals, particular tropism for lymphoid tissue and organs, related acquired immunosuppression and secondary infections) [9, 10].

Circoviruses are tiny non-enveloped icosahedral viruses (15- 24 nm in diameter), the smallest known animal viruses with a single-stranded circular DNA of 1.76 to 2.31 kb [11, 12]. Like most other members of the newly proposed Circoviridae family, PiCV is characterized by typical basophilic globular inclusions in light microscopy [3, 13, 14].

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PiCV primarily affects young pigeons 2 months to 1year of age. Spreads appear to be mainly horizontal; occurrence and possible significance of vertical transmission are known. Gross and histological features of infection with this virus have not been well characterized. Abdominal enlargement, gall bladder congestion and yellowish fluid in the air sacs were observed in nestlings from a typical "black spot" outbreak, with histological lesions strongly suggestive of circovirus infection, in birds 10 to 20 days old, including necrosis of the bursa, feather epidermal collar and the oral epithelium [15]. In a second report, adult canaries that died after a short illness had only pinpoint hemorrhages in their muscles [4].

The present study reported an outbreak of PiCV in Libya in a loft of racing pigeons in Western Libya.

MATERIALS AND METHODS

A large rearing loft for racing pigeons in El-Nekat El-Khams-Western Libya suffered from high mortalities among the reared pigeons. All pigeons were 4-8 week old or less. They were submitted for necropsy to the Department of Pathology of Faculty of Veterinary Medicine and Agricultural Sciences during the winter of 2008. The reported clinical signs were not specific, both male and female were affected. Clinical signs include loss of body weight, diarrhea, lethargy, listeless and some showed paralysis of legs and wings and increased mortality. Mortality rates varied from 5 to 40% among flocks in the loft and death usually occurred 2 to 4 days after the first signs and the spread among the flock was rapid.

The last breeding season of the loft had been characterized by 50% total mortality in both adults and younger birds. The latter were typically affected during the weaning period. The adults died after 1 or 2 days of anorexia and lethargy.

Case Selection: Twenty-five cases (five birds per flock) for a total of 5 flocks were selected for further study based on the severity of clinical signs and necropsy findings then on the presence of basophilic globular inclusions suggestive of Circovirus-like virus in the bursa of Fabricius.

Twenty cases were young in the fledging period (either healthy or presenting non-specific clinical signs of variable severity) and 5 were mature breeder pigeons. All birds, except a naturally dead one, were humanely killed by intravenous administration of sodium pentobarbital (vena ulnaris). Complete necropsy was immediately performed and samples of the bursa of Fabricius and other internal organs were systematically collected for histopathological examination.

Pathological Studies: At necropsy all birds were weighted and through PM examination were done and any PM changes were recorded. Specimens for histopathological examination were taken primarily from bursae and other body organs then fixed in 10% buffered formalin, routinely processed and embedded in paraffin blocks. Sections 4 μm thick were cut, mounted on glass slides and stained with haematoxylin and eosin and with Feulgen stain [16].

Electron Microscopical Examination: Other specimens from the corresponding bursae were fixed in 2.5% phosphate-buffered glutaraldehyde, post-fixed in osmium tetroxide and processed, embedded and sectioned according to standard procedures for transmission electron microscopic examination [17].

***In Situ* Detection of Apoptosis in Bursal Follicular Cells:** *In situ* apoptosis detection was performed on sections of bursa of Fabricius of five pigeons showing evidence of circovirus infection. Three other pigeons of the same age with no evidence of circovirus infection or other concurrent disease were used to assess the physiological apoptotic process in normal bursa of Fabricius. Qualitative evaluation of the number, size and distribution of foci containing positive nuclei and their fragments was performed for each bursa of Fabricius. Apoptotic cells in tissue sections of the bursa of Fabricius were detected by the *in-situ* cell death detection kit, The DEdEnd™ Colorimetric TUNEL System (Promega, G7131, G7132, USA) according to the manufacturer's instructions. This system depends on end-labeling the fragmented DNA of apoptotic cells using a modified TUNEL assay.

RESULTS

PiCV was diagnosed in 19 pigeons from five different lofts. All were nearly 2 months old or younger. PiCV was never detected in mature pigeons. Histological diagnosis of PiCV was based on detection of viral inclusion bodies in the cytoplasm of macrophages present in the bursa of Fabricius and other lymphoid organs and tissues.

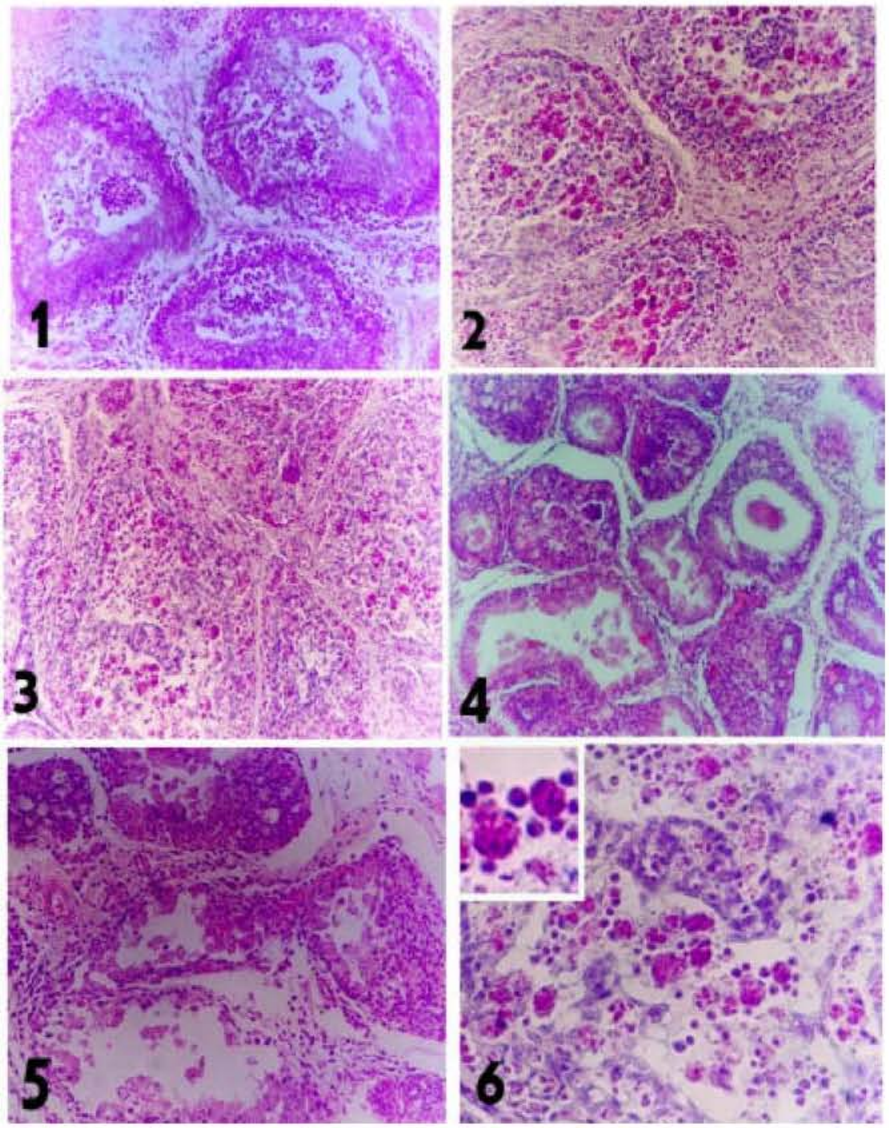

Fig. 1-6: Histopathological features of bursal follicles of PiCV infected pigeon showing: 1: Marked lymphocytic depletion with increased number of pychnotic lmphocytes. 2,3: numerous botryoid inclusions. 4: Cystic degeneration and lobular atrophy together with moderat number of inclusions. 5: Diffuse necrosis of bursal issue. 6: Multifocal replacement of the bursal tissue with inclusion bodies which were sparse in the rest of the bursal tissue. (H&E, X 200 and 400).

Light Microscopic Examination: Histological alterations in different body organs from different birds revealed randomly dispersed inflammatory lymphocytic or heterophilic infiltrates in lungs, liver or myocardia of individual birds. While, distinct histopathological changes were observed in lymphoreticular tissue, especially bursa of Fabricius and spleen. Those changes ranged from lymphofollicular hyperplasia, lymphocytic depletion and histocytosis. Lymphofollicular hyperplasia was sometimes seen in spleen with variable degrees of discrete lymphocellular necrosis. Bursal follicles were particularly damaged and showed cystic degeneration. The most conspicuous lesion was the presence of intracytoplasmic inclusions appeared as botryoid crystalline globular or coarse granular basophilic bodies of various sizes in the follicular cells. Some of these inclusions exceed the size of neighboring cells were evident within the cytoplasm of follicular cells and splenic macrophage. Inclusions could also be seen in gut and bronchial associated lymphofollicluar tissues.

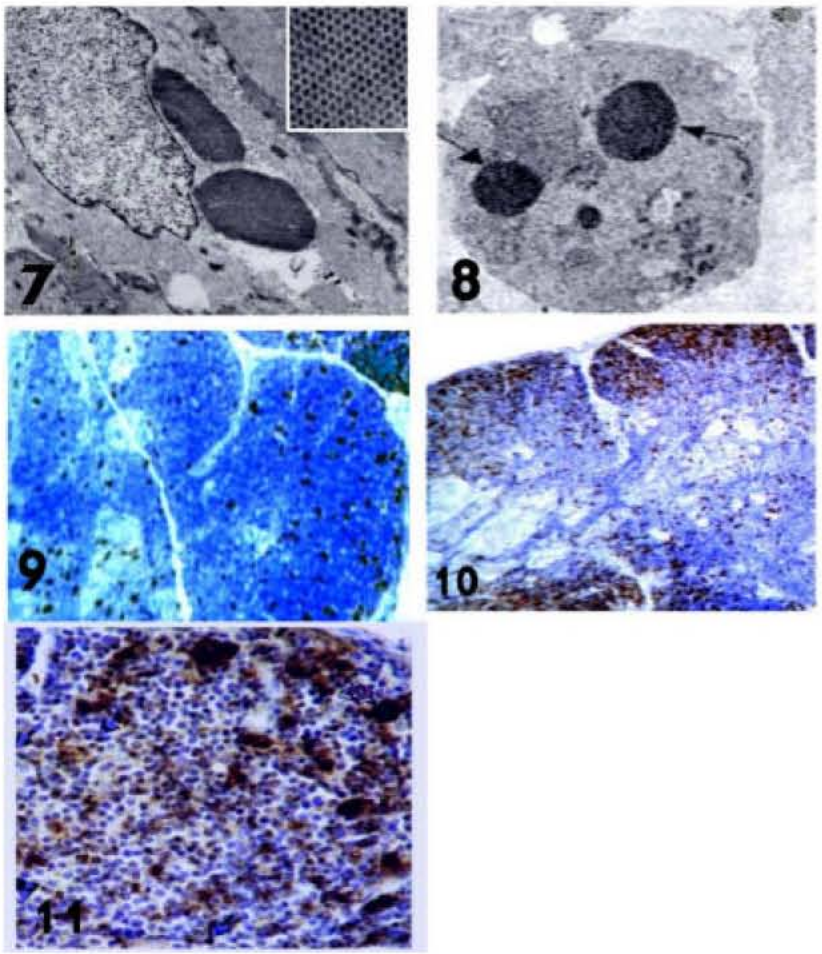

Fig. 7-11: Transmission electron microscopy of PiCV infected bursa revealing: 7: Membrane-bound paracrystalline arrays of virus particles with icosahedral morphology. 8: Nuclear fragmentations of the follicular cells forming several apoptotic bodies. 9: Few isolated apoptotic cells scattered in the follicular cortex of the bursa of Fabricius from uninfected birds. 10, 11: Large number of isolated apoptotic cells and extensive foci of several cell fragments either free or in the cytoplasm of macrophage

Generally, according to the bursal changes; three groups of birds were defined. In the first group, the bursal architecture was normal, but the parenchyma of the follicles showed marked lymphocytic depletion with increase in pychnotic lmphocytes (Figure 1) and multiple botrid inclusions (Figure 2,3). In the second group bursal follicles were particularly damaged and showed cystic degeneration and lobular atrophy together with moderate number of inclusions (Figure 4). While, the third group revealed diffuse necrosis of bursal issue (Figure 5) as well as multifocal replacement of the bursal tissue with inclusion bodies which were sparse in the rest of the bursal tissue (Figure 6). However, no histological evidence of specific concomitant viral infection other than PiCV (i.e. particularly adenoviral and/or herpesviral infections) were detected or suspected by microscopic examinations. Moreover, the great majority of these infectious lesions could be related to bacterial ones.

Mild catarrhal enteritis was observed along the intestinal tract, especially at its beginning with evident botryoid inclusions in the cytoplasm of smooth muscles of the muscular layer.

Electron Microscopical Examination: Inclusion bodies were never detected by TEM in the bursa of Fabricius of pigeons in which circovirus inclusion bodies were not already detected by light microscopy.

Examination of bursae by transmission electron microscopy revealed irregular oval or sometimes angular inclusions and contained electron dense, partially

membrane-bound paracrystalline arrays of 16±2 nm diameter virus particles with icosahedral morphology (Figure 7). In some follicular cells a few virus particles, both in loose aggregates and in a paracrystalline arrangement were associated with electron-dense granular material. However, nuclear fragmentations of the follicular cells were more conspicuous in most of the examined bursae forming several apoptotic bodies (Figure 8).

***In Situ* Detection of Apoptosis:** A few isolated apoptotic cells scattered essentially in the follicular cortex of the bursa of Fabricius from uninfected birds was noticed (Figure 9). A moderate to large number of isolated apoptotic cells and locally extensive foci of several cell fragments (Figure 10, 11) were observed either free or more often found in the cytoplasm of macrophage.

DISCUSSION

Circovirus infection in pigeons was first reported in the USA during 1990 [3]. The present work described PiCV in Western-Libya. Specific diagnosis of PiCV was based on histopathological observations of the bursa of Fabricius and the potential concurrent lesions of other primary and secondary lymphoid tissues. Three groups of birds were defined on the basis of histopathological lesions observed in their bursae. These three groups could be considered as different stages in the chronological progression of PiCV-associated lesions. Thus, in the early stage of infection, viral inclusion bodies were only detected in bursal macrophages of bursal follicles. Normal bursal architecture and cellularity, as compared with pigeons of the same age with no evidence of circovirus infection or concurrent disease, were conserved. However, a marked increase in pyknotic lymphocytes was observed. These features were highly suggestive of the primary burso-tropism of pigeon circovirus. Subsequently, bursal lesions progressed to cystic degeneration and diffuse lobular atrophy, with lymphocytic corticomedullary depletion. Viral inclusion bodies were also found in mononuclear cells in other lymphoid tissues with concomitant progressive lymphoid depletion and histiocytic infiltration. The later lesions led in some cases to difficulty in determining the identity of cells in which the basophilic globular inclusions were found because of the autolysis of cells. All of these pathological lesions were suggestive for immunosupression and predisposition of these birds to various secondary infectious agents as the observed clinical signs and other lesions were reflective for other concurrent infections. Similar findings of immunosuppression with resultant secondary infectious agents have been reported in association with chicken anemia virus (CAV) [18], Psittacine beak and feather disease (PBFD) virus [19, 20] and Circovirus [21].

Typical basophilic inclusion bodies were found in the cytoplasm of mononuclear phagocytic cells (exclusively in primary and secondary lymphoid organs). As the bursa of Fabricius invariably contained macrophages with inclusion bodies, it was used to detect the infection in affected birds. These morphologic features were consistent with previous reports on PiCV [9, 10, 13]. Knowledge about the pathogenesis of PiCV is limited. However, the disease shows similarities with other diseases associated with circovirus infection in animals, such as the preferential lymphoid tissue tropism of the viruses and subsequent related immunosuppression [9]. In this study, all circovirus-infected birds were from 4 weeks to 8 weeks of age, i.e. the post weaning period. This age range may be indicative of the time period in which the fully developed bursa of Fabricius represents a suitable target for virus invasion and proliferation.

Circovirus lesions in aviary birds (psittacines, pigeons, finches) are usually seen in the skin and lymphoid tissues, where characteristic multiple globular inclusions are seen in the cytoplasm of mononuclear cells of these organs [19, 21, 22, 23]. However, the opportunistic and secondary infections are consistent with humoral and cell-mediated impaired immune functions associated with severe diffuse lymphocyte depletion of primary and secondary lymphoid organs. Individual bird differences in susceptibility could equally explain the presence of some degree of lymphoid hyperplasia in lymphoid tissues of certain infected birds. Moreover, if such a lymphoid hyperplasia represents a true immune reaction against circoviral infection and could thus afford a protection or even an elimination of the viral infection, remains yet to be determined [21]. One of the striking features of PiCV is the absence of an obvious inflammatory process, particularly in the early stage of the disease. The bursa of Fabricius in Circovirus infected pigeons displayed specific morphological features of lymphocytic death by apoptosis expressed by moderate to large number of TUNEL-positive apoptotic cells were observed among the bursal cells of infected birds. The later represented by cell shrinkage, nuclear and cellular fragmentation and formation of small rounded cellular fragments by electron microscopy. However, similar findings were noticed by Majno and Joris [24].

These above mentioned features were also observed in non-bursal lymphoid tissues undergoing lymphocytic depletion [21]. Detection of *in situ* apoptosis by the TUNEL method revealed a dramatic increase of the apoptotic process in the bursal lymphocellular populations of Circovirus infected pigeons as compared with the physiological apoptotic process in normal bursa of Fabricius of uninfected age-matched birds. These findings suggested that PiCV-induced lymphoid depletion and subsequent immunosuppression may be caused at least partly by activation of apoptosis in lymphocytes. However, inoculation studies and associated evaluation of immune function would be necessary to confirm these findings [21]. The DNA fragments of apoptotic cells could be detected at the cellular level on tissue sections by the TUNEL assay. This procedure enables specific immunolabelling of apoptotic cells and their quantification on routinely processed tissues. Apoptosis in primary lymphoid organs includes the elimination of non-functional or autoreactive lymphoid cells [25], a process that may account for the physiological presence of only a few apoptotic cells scattered in the cortex of bursal follicles in the healthy birds used as circovirus-free controls in our study. Apoptosis has also been found to be the mechanism of cell death in several viral infections, particularly in avian species. Recent *in vitro* and *in vivo* studies have shown that apoptosis was primarily involved as the main pathological event in infectious bursal disease [26] Newcastle disease [27], Marek's disease [28]. Ultrastructural investigation of PBFD virus-infected cells revealed morphological alterations typical of apoptosis [29]. Such opinion was in agreement with Stevenson *et al.* [21]. However, it is not clear whether apoptosis is associated with infection of cells with pigeon circovirus or results from some other process during infection not directly related to viral replication. Conversely, apoptosis may be a cellular defense mechanism to prevent virus spread by host-cell death, in which case infected cells would be removed without inducing life-threatening processes, i.e. inflammatory reactions.

Experimental induction study of the disease is needed to confirm full characterization of the chronological progression of lesions and the primary apoptosis dependant mechanism of pathogenesity.

It was concluded that further investigations are needed in several lofts in Western Libya to evaluate the prevalence of PiCV. Pathological findings suggest that cell death by apoptosis is involved in pigeon circovirus-induced damage to lymphoid organs.

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