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ABSTRACT. Psittacine beak and feather disease (PBFD) affects a large number of Psittaciformes species. In this study, five White Cockatoo parrots (*Cacatua alba*) with clinical signs of PBFD were examined. After euthanasia, a full necropsy of parrots was performed and organs with macroscopic changes were sampled for routine histopathological evaluation. To confirm the presence of psittacine beak and feather disease virus (PBFDv), feather samples were analyzed with the PCR method. Sequence analysis of the obtained PCR products indicated their close relationship (99%) to other PBFDv isolates. Six variable nucleotide sites were discovered, two missense and four silent mutations. This paper presents the evidence of new PBFDv sequence in Cockatoo species.

Keywords: PBFD, PCR diagnostic, nucleotide sequence, *Cacatua alba*

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INTRODUCTION

Psittacine beak and feather disease (PBFD) is a highly contagious disease of many avian species primarily from the order Psittaciformes and presents the major problem of parrots worldwide (Khalesi et al., 2005). The disease is described in more than 60 parrot species, but it has been proposed that all species are susceptible (Rubinstein and Lightfoot, 2012), equally in captive and wild parrot population (Ritchie et al., 2003). Till very recently the causative agent of PBFD, psittacine beak and feather disease virus (PBFDv), was thought to be restricted to within avian orders such as the Psittaciformes. However, recent studies have also identified PBFDv in non-psittaciformes species - *Ninox strenua* (Sarker et al., 2015a) and *Merops ornatus* (Sarker et al., 2015b).

PBFDv belongs to genus *Circovirus*, family *Circoviridae* (Niagro et al., 1998; Julian et al., 2013), is highly resistant and one of the smallest known viruses. The viral genome has a single-stranded DNA of about ~2kb in size with two major open reading frames (ORFs), which encode proteins involved in the replication of the virus (V1), and its encapsidation (C1) (Ogawa et al., 2010). PBFDv has a high affinity to the lymphoid tissue cells causing strong immunosuppression (Todd, 2000).

Experimentally, PBFD has been reproduced by the inoculation of PBFDv into susceptible birds in different ways, including *per os*, intracloacal, intranasal, intraconjunctival, subcutaneous, intramuscular and respiratory routes (Khalesi, 2007). Extremely high concentrations of the virus have been detected in the faeces and feather dust that are believed to be the main source of infection. Vertical transmission of the virus is uncertain in the natural transmission of disease, but PBFDv has the potential to be transmitted vertically (Rahaus et al., 2008).

The clinical signs vary depending on the species and age of infected birds (Gerlach, 2004), but new cases show that they are strictly host related (Robino et al., 2015). The occurrence and the degree of observed clinical signs correlate strongly with viral load (Regnard et al., 2015). PBFD is potentially fatal and can manifest in the peracute, acute, or chronic form of infection, with the latter as the most frequent, known as the "classical form".

PCR, based on the detection of conserved ORF V1 region (Ypelaar et al., 1991; Ritchie et al., 2003), is used throughout the world to diagnose diseases, since it is much more reliable than other diagnostic methods, such as HA or HI (Khalesi, 2007). The virus is present in blood shortly after infection (Latimer et al., 1991) and has a tropism to the organs of the immune system (especially to the thymus and bursa Fabricius) and to rapidly proliferating cells, such as epithelial cells of feathers and feather follicles. Thus, it is not surprising that feathers have proved to be the most appropriate as samples for PCR analysis (Hess et al., 2004).

There is no effective antiviral treatment for PBFD. The inability to propagate PBFDv *in vitro* hampers the production of a protective vaccine.

The aim of this study was to examine the presence of PBFDv in Cockatoo parrots in the acute form of the disease, as well as to perform histopathology analyses of infected birds and virus sequencing.

MATERIALS AND METHODS
Animals – sample collection

Samples were collected from White Cockatoo parrots (*Cacatua alba*). Five parrots (4-month-old) from a private aviary were brought to the University Hospital for Small Animals of the Faculty of Veterinary Medicine University of Belgrade in December 2013. Gender was determined (2 males and 3 females) using methodology described by *Vucicevic* et al. (2016) All birds were confirmed to be free of parasites, pathogenic bacteria or fungi. Examined birds had clinical signs of an acute form of PBFD. After molecular confirmation of PBFDv by PCR, the owner decided to euthanize the animals, in accordance with the national law and regulations.

Pathology and Histopathology

A full necropsy of parrots was performed and organs with macroscopic changes were sampled and fixed in 10% neutral buffered formalin for routine histopathological evaluation. Tissues for light microscopy were processed in automatic tissue processor LEICA TP1020, embedded in paraffin, and cut at 4 µm. Initial sections were stained with hematoxylin and eosin (HE) and analyzed by light microscope (BX51, Olympus Optical, Japan). Digital imag-

es were made using an optical microscope Olympus BX51 with digital camera Olympus Color View III.

Molecular detection

Three growing feathers with irregular growth were pulled out from each bird and used for DNA isolation. Feathers were used for analysis because this type of sample appeared to be the most appropriate according to the study of Hess et al. (2004). Isolation was performed using commercial set "KAPA Express Extract Kit" (Cat. No KK7152, Kapa Biosystems, Cape Town, South Africa) according to manufacturer's instructions.

For PCR amplification, we used primers designed by Ypelaar et al. (1991) (5'-AACCCTACAGACGG-CGAG-3' forward and 5'-GTCACAGTCCTCCTTG-TACC-3' reverse). PCR reaction mix was prepared using commercial set KAPA2G Robust HotStart ReadyMix (Cat. No KK7152, Kapa Biosystems, Cape Town, South Africa) according to manufacturer's instructions. The thermal protocol involved 3 min of initial denaturation at 95ºC, followed by 45 cycles of denaturation (95ºC, 15 sec), primer annealing (59ºC, 15 sec), extension (72ºC, 15 sec), and a final extension step at 72ºC for 8 min. PCR products were visualized with UV light after staining the 2% agarose gel with ethidium bromide. A commercial O'RangeRul-erTM 100bp DNA Ladder (Thermo Scientific) was used as size marker.

DNA obtained from PBFD positive bird was used as a positive control for PCR. As a negative control, we used DNA/RNA free water.

DNA sequencing

Obtained PCR products were directly sequenced in two directions using the BigDye® Terminator method in an ABI 3730XL automatic DNA sequencer (Macrogen Europe, The Netherlands). Sequence similarity analysis was performed using the BioEdit version 7.2.5 and Clustal W software.

RESULTS
Necropsy

Fig 1: Wing feather dystrophy

Fig 2: a) Delaminations of the beak; b) Inflammation of duodenum

In all parrots with symptoms of PBFD, cachexia was evident. The parrots had atrophy of the pectoral muscle and prominent keel bone. In all parrots, symmetrical feather loss was observed, especially on the wings and tail (Figure 1). Newly grown feathers were small and bended. Abnormalities like clubbed, constricted or otherwise deformed feathers were observed. Fractures and delaminations of the beak were also recorded (Figure 2a). Two parrots had duodenitis (Figure 2b) and focal necrosis of the liver.

Histopathology

Intracellular oedema, apoptosis or necrosis of the keratinocytes were evident in histopathological observation of the skin. Hyperkeratotic changes were present on the epithelium surface. Apoptotic keratino-
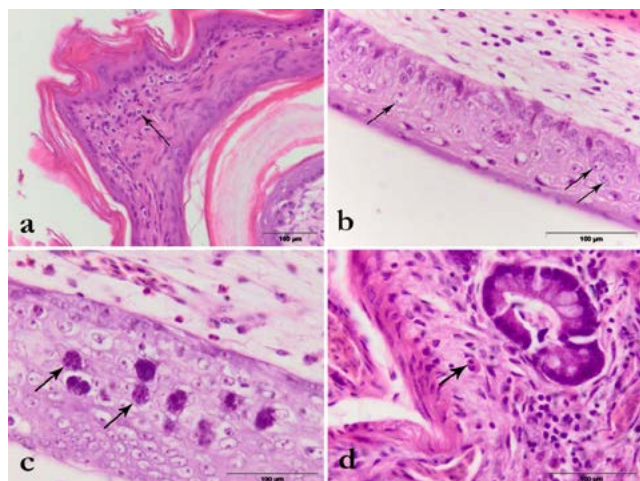

Fig 3: Microscopic changes, PBFD: a) Skin, Parrot, "halo" zone around the keratinocytes as a sign of apoptotic changes, hyperkeratotic deposits on surface of the skin, HE, 600x; b) Feather epithelium, Parrot, Intranuclear inclusions in keratinocytes (arrow), HE, 600x; c) Feather epithelium, Parrot, Macrophages in epithelium with numerous cytoplasmatic inclusion (arrow), HE, 1000x; d) Intestine, Parrot, Lamina propria infiltrated with lymphocytes and macrophages. Cytoplasmatic inclusion are visible in macrophages (arrow), HE, 1000x

cytes were recognizable by "halo" zone (Figure 3a). In keratinocytes of the feather follicles, intranuclear inclusions were visible (Figure 3b) as well as some nuclei that were vacuolated and transparent. The epithelium of the plucked feather was infiltrated with macrophages. Numerous intracytoplasmic inclusion

bodies were observed within macrophages (Figure 3c). Necrotic pulp with an intense infiltrate of heterophilic granulocytes was often visible on feather sections. Dense infiltrate of macrophages, lymphocytes and plasma cells were present around feather follicles (perifolliculitis).

The small intestine lamina propria was intensively infiltrated with macrophages and lymphocytes. Macrophages with cytoplasmic inclusion bodies were observed in hematoxylin and eosin stained sections of lamina propria of the intestine (Figure 3d).

The histopathological examination of the liver revealed the presence of necrotic foci surrounded by a mononuclear inflammatory infiltrate. The majority of hepatocytes were with intracellular oedema.

Molecular analysis

PBFDv was diagnosed in all tested birds based on the detection of viral nucleic acid in feathers. The obtained amplified products were of the same size as the positive control represented by the product derived from the bird with previously diagnosed PBFD using PCR method. The analysis of the gel showed the presence of amplicons size of 717 bp (Figure 4), indicating that all examined animals were indeed infected with the PBFDv.

The obtained sequences from all five samples (GenBank Accession Number KJ413143) were identical and had 99% nucleotide similarity with PBF-
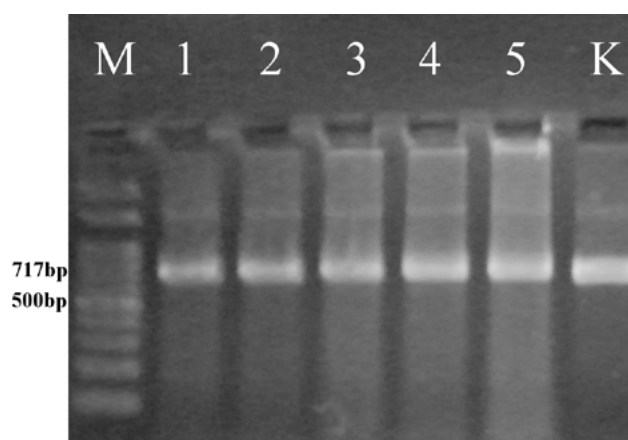

Fig 4: Ethidium bromide stained agarose gel showing PCR products amplified from feather samples taken from suspicious birds. M – 100bp DNA Ladder, 1 - 5– *Cacatua alba* (tested birds), K – Positive control

Fig 5: Nucleotide polymorphisms in 717 bp long sequence of PBFDv rep gene: G sequence corresponds to the complete PBFDv genome from positions 301-730 bp published by Varsani et al., 2013 (JX221037) (28). S is part of the sequence newly described in this work (GenBank Accession Number KJ413143). Numbers correspond to variable sites and dots indicate an identical nucleotide as in the complete genome sequence

Dv sequence already deposited in GenBank (Accession Number JX221037, Latimer et al., 1991). Six variable nucleotides were observed at positions 321, 359, 524, 585, 629 and 725 of the complete PBFDv genome (Figure 5). Two of them are missense mutations and the rest are silent. Mutation at the position 585 causes replacement of arginine by cysteine, whilst mutation at the position 725 results in aspartic acid being substituted by glutamic acid.

DISCUSSION

Cockatoos analyzed in this study were hatched in an incubator and fed by hand. By the age of two months, cockatoos had no clinical signs and the precise date of infection is unknown.

Cachexia and atrophy of the pectoral muscle were the results of the inability of food intake due to the beak damage. Symmetrical feather loss was observed especially on the wings and tail that corresponds to previous findings in which loss of feathers usually starts foremost at the tail (Rosskopf and Woerpel, 1996). Newly grown feathers were small, bended and clubbed due to feather dystrophy caused by PBFDv as already had been reported by other authors (Kondiah, 2008).

The inclusions found in feathers and epithelial cells indicate their presence in the tissues in which the virus persists even in the absence of clinical symptoms and is in accordance with previously find-ings (Hess et al., 2004). Findings of intranuclear and intracytoplasmic inclusion bodies in epithelial cells suggest that PBFDv is epitheliotropic in feathers and follicles (Latimer et al., 1990). Macrophages may become infected during phagocytic removal of virus-containing epithelial detritus and consequently, intracytoplasmic inclusions of macrophages contain PBFD viral antigen (Latimer et al., 1990). Viral inclusions that were observed in macrophages of lamina propria of alimentary tract may also be noted in many other organs such as liver, thymus, spleen, parathyroid gland and bursa of Fabricius (Ramis et al., 1998, Sa et al., 2014).

According to our results the feather abnormalities seen in PBFD-affected birds seems to be the consequence of apoptotic, necrotic and hyperkeratotic changes or hyperplasia of the epithelial cells as already were reported by other authors (Kondiah, 2008; Robino et al., 2015). Some authors suggested that necrotic changes causing feather dystrophy are the consequence of secondary infections due to PBFDv's immunodeficient properties (Hattingh, 2009).

Unfortunately, the opportunity to test if parents of the examined birds or other birds from the same aviary carry the virus was missed although the owner has stated that other birds in his aviary did not show any symptoms that may indicate PBFD. Rahaus and Wolff (2003) found a subpopulation of birds that carried the virus but were asymptomatic. These birds could be in the viral incubation phase, has a subclini-

cal form of infection or carried the virus chronically (Rahaus and Wolff, 2003; Hess et al., 2004). In order to prevent the spread of PBFDv inside captive bird populations, the researchers recommended effective implementation of monitoring and quarantine. Additionally, this monitoring could help in detecting birds in the viral incubation phase (Rahaus and Wolff, 2003).

For more than 20 years, DNA analysis is the method of choice for diagnosis of PBFDv in live birds (Hess et al., 2004). The PBFDv infections can be detected using the PCR assay after the DNA isolation from feathers. The protocol used for the isolation of DNA from feather samples is rapid and non-invasive. The PCR most likely detects viral particles within the pulp or sheath of sick birds' feathers. There are numerous factors that greatly contribute to the spread of infection: high virus resistance, uncontrolled (and often illegal) importation of parrots from other countries (Bosnjak et al., 2013), the presence of the virus in the region (Gottstein et al., 2005), and the absence of legislation to regulate the mandatory quarantine on import of birds from countries that are not free from the PBFDv (Julian et al., 2013). Regions of the world that import parrots may be playing a major role in PBFDv dissemination and in its accelerated genetic diversification within captive birds (Harkins et al., 2014). Since birds can be virus carriers even though they show no symptoms (Rahaus and Wolff, 2003, Hess et al., 2004), molecular genetic diagnostic procedures should become part of the regular and mandatory measures during quarantine (Araujo et al., 2015; Hakimuddin et al., 2016) of birds that are imported into a country where PBFD has not previously been reported. Such information is necessary in order to form recommendations for legislative regulation of PBFD control, in order to prevent future outbreaks.

The mutations we found in this study could be viral adaptations to a specific host or regional differences of the strains. The importance of the existence of difference of nucleotide sequences is not known and there is little evidence to support a relationship between the genetic variation and the regional distribution of the isolates, or that there are differences in pathogenicity, antigenicity, or any other physicochemical characteristics of PBFDv (Bassami et al., 2001). Although the PBFDv is genetically diverse and demonstrates a high mutation rate (Julian et al., 2013; Sarker et al., 2014), it is still relatively antigenically conserved and so far is revealed only a few different serological strains (Khalesi et al., 2005; Shearer et al., 2008). The role of high degree of genetic variation in the evolution of PBFDv in cockatoos and in ssDNA virus replication remains unclear. In the case of PBFDv, it is probably a mechanism used to enhance replicative capacity rather than immune escape since all known PBFDv so far studied have been antigenically similar. Viral recombination and mutation are the most important evolutionary mechanisms that affect pathogen and host diversity and enable their adaptation (Awadalla, 2003; Eastwood, 2014). The ability to change the host may be an important mechanism for sustainable PBFDv replicative competency and may be the main reason why the virulence is maintained in this circovirus species compared to others (Sarker et al., 2014).

In conclusion, the results presented in this study are of significance for epidemiological studies aiming to investigate the prevalence of PBFDv infections in birds. More extensive research is also necessary to be carried out in order to find out if the virus sequence obtained in this study exists anywhere else. Virulence of this virus should be tested since two of the six mutations affect protein sequence (missense mutations) and the fact that there is a report that new genotypes of the virus have shown dramatic differences in virulence and resistance in the environment (Sa et al., 2014).

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