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Radiology versus RT-PCR for detection of proventricular dilatation disease in psittacines

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Abstract: Proventricular dilatation disease (PDD) is a potentially fatal disease of psittacine birds. *Avian bornavirus* (ABV), the causative agent of PDD, causes inflammation of the central, peripheral, and autonomic nervous systems. Clinically, birds affected by PDD present with gastrointestinal tract dysfunction, neurological symptoms, or both. In this study, we evaluated the presence of proventriculus dilation and ABV in 40 birds (cockatiel, n = 12; budgerigar, n = 28), submitted to the veterinary clinic, by radiology and RT-PCR. Lateral and ventrodorsal radiographs and feather samples were taken from 36 live birds including the cockatiel and budgerigar, as well as samples of proventriculus from four autopsied birds. After reviewing the radiographs, 55.5% of the evaluated birds had proventriculus dilatation, and according to RT-PCR results, 47.5% of samples were positive. In most cases, radiology and RT-PCR results matched together very well, but in some cases, differences were observed. These discrepancies between the results of these two assays require further studies. Based on the results of this study, the prevalence of ABV was high in the region, and the importance of hygiene and testing of new birds should be emphasized. Simultaneous evaluation of the bird with these two methods is helpful for the diagnosis of PDD.

Key words: Avian bornavirus, proventriculus dilatation, RT-PCR, radiology, cockatiel *(Nymphicus hollandicus)*, budgerigar *(Melopsittacus undulatus)*

1. Introduction

For the first time in North America and Europe, disorders of the gastrointestinal tract and nervous system which caused proventriculus dilatation in macaws were described [1,2], and Avian Bornavirus (ABV) was identified as the main etiologic agent of this disorder in 2008 by using an electronic microscope [3,4]. This novel virus is a subset of the family Bornaviridae. This single-stranded RNA virus has at least 15 genotypes of which parrot Borna Virus 2 and 4 are more common in parrots [3,5]. The virus can infect humans, mammals, reptiles, and many orders of birds, and so far, it has infected about 20% of 398 parrot species [6]. PDD is characterized by dilation of the proventriculus, in most cases, associated with microscopic changes such as lymphoplasmacytic ganglioneuritis involving the gastrointestinal tract, nonsuppurative encephalomyelitis, peripheral neuritis, ganglionitis, myocarditis, adrenalitis, and lesions in the eye and skin [7–9]. This fatal virus affects the autonomic system. Then, causes disorders in the upper and middle gastrointestinal tract [3] which are manifested by weight loss, undigested seed in the droppings, regurgitation, crop stasis, and lack of energy and appetite [10]. Moreover, central nervous system disorders are manifested by convulsion, blindness, and loss of balance which sometimes leads to paralysis [8,11]. De Araujo et al., admitted cardiac involvement in PDD cases as a possible cause of sudden death in psittacines [12]. PDD has a very variable incubation period, and this factor has created a great challenge in the control strategies of the disease [4,13].

Diagnosis of PDD is based on history, clinical signs, and ancillary diagnostic tests. Several researchers acknowledged radiology as a useful diagnostic test in PDD cases [11,14,15]. ABV genome can be detected by RT-PCR using samples from live and dead birds [16–19]. Further tests for the detection of PDD infection in birds are immunohistochemistry, virus isolation in quail cell lines [18,20] or duck embryo fibroblasts [21], in situ hybridization [22], and serological methods [19–23]. For this study, we used radiology and RT-PCR as common and available tests for monitoring and ante-mortem diagnosis of PDD in birds.

Due to the beauty and high intelligence of psittacine, these birds have many fans in most countries of the

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world. An outbreak of a disease such as PDD can cause great economic and psychological damage to the owners. Regarding poor prognosis and no specific treatment for this disease, it is very necessary to identify and control it. To the best of our knowledge, no study has been conducted on PDD in Iran. The purpose of the study reported here is to investigate the presence of proventricular dilatation and ABV genome in PDD-suspected psittacines referred to a veterinary clinic by radiology and RT-PCR, respectively.

2. Materials and methods

2.1. Ethics statement: Sample collection from the birds and carcasses was accomplished for medical purposes and done only with the owner's permission and consent. Sample collection was performed by the recommendation of the ethics committee of Ferdowsi University of Mashhad (IR.UM.REC.1398.112).

2.2. Sample collection: In this study, we collected tissue samples including proventriculus and feather from 40 psittacines of two species (budgerigar and cockatiel) which were referred to the specialized veterinary hospital of Ferdowsi University in Khorasan Razavi of Iran from September to December 2020. A complete clinical examination was performed on the birds. The presence of undigested seeds in droppings, regurgitation, emaciation, and neurologic symptoms were noted in the case history. After clinical evaluation, the birds were subjected to radiology and molecular detection of the ABV genome.

2.3. Radiology: Whole-body radiographs of 36 live psittacines were evaluated for the symptoms of proventricular dilation. High-quality right lateral and ventrodorsal radiographic projections were obtained using a commercial x-ray unit (Siemens, Multix top, Erlangen, Germany), and flat panel X-ray detector (Perkinelmer Xrpad2 4336 Hwc-m, 65396 Walluf, Germany). The exposure factors were 52 kVp and 4.5 mAs. The focus film distance was 100 cm.

The primary inclusion criterion was a right lateral whole-body radiography. The following ratio was calculated from the lateral radiographic images by the formula: proventriculus diameter/dorsoventral height of the keel [24]. According to the measured ratio, the birds were assigned into one of the following groups: 1. no gastric dilatation, 2. suspected of dilatation, and 3. presence of gastric dilatation (Table 1).

2.4. Molecular detection of ABV: After obtaining the verbal consent of bird owners, samples of feathers and proventriculus were taken from 36 live and four wasted birds, respectively, to evaluate the presence of the bornavirus Matrix gene. Several feathers were taken from the bird's chest. Then, the hollow shaft (calamus) of each feather was separated using a sterilized scalpel. The sample was transferred to a microtube and then froze at –80 °C. For carcasses, samples were taken from proventriculus tissues, transferred to a microtube, and then stored at –80 °C for further analysis.

Viral RNA was extracted from tissues by using an RNA extraction kit (Sinaclon, Iran) according to the manufacturer's instructions. The extracted RNA was then converted to cDNA-by-cDNA synthesis kit (Parstous, Iran) according to the guidelines recommended by the manufacturer, under the following conditions: 25 °C for 10 min, 47 °C for 60 min, and 85 °C for 5 min. The cDNA served as a template for PCR assay targeting the Matrix (M) gene. Primers were selected based on a previously published article [25]. PCR was carried out with a master mix of Biofact Company (South Korea) and a pair of the following primers for amplification of a 350 bp sequence: ABV M F (5' -GGTAATTGTTCCTGGATGG- 3'); ABV M R (5' -ACACCAATGTTCCGAAGACG- 3'). The last step of PCR was to split the PCR products based on their size by electrophoresis in an agarose gel.

2.5. Statistical analysis: Chi-square and Fischer's exact tests were used to compare the frequency of liquefaction of feces, vomiting, the existence of undigested seeds, and neurological signs between PCR-positive and PCR-negative birds. The mentioned statistical tests were also applied to evaluate the association of age categories and species with PCR and radiology results.

3. Results

According to radiographs taken from 36 birds, 20 birds had proventriculus dilatation, three birds were suspected of proventriculus dilatation, and in seven birds we were not able to measure the diameter of the proventriculus and the height of the keel. Other samples (n = 6) had no gastric dilation (Figures 1, 2, and 3) (Table 2). Proventriculus dilatation was observed more commonly in Budgerigars than in Cockatiels ($p = 0.032$). Results of radiographs showed that there is no significant difference in the prevalence of proventriculus dilatation between birds <2 months old and >2 months old ($p = 0.26$). Regarding these results, the definitive diagnosis of PDD is not possible using radiographs, and various factors such as fungal and bacterial infections, poisoning with toxins and metals, and the presence of foreign bodies may cause dilatation of the proventriculus. It is noteworthy to mention that evidence of heavy metal poisoning was not observed in the radiographs of these cases. Therefore, we rule out the possibility of heavy metal poisoning in these birds.

Based on RT-PCR results, 40 birds were sampled, and 19 birds tested positive (Table 2). These results were obtained from 17 feather samples and 2 proventriculus samples. A total of 12 out of 28 feather samples (42.8%) of budgerigars were positive in RT-PCR. For cockatiels, 5 out of 8 feather samples (62.5%), and 2 out of 4 proventriculus

Table 1. The birds were divided into one of the three following groups based on the ratio obtained in the lateral radiographic images.

Ratio (proventriculus diameter/dorsoventral height of the keel)	Gastric sign
0.200–0.476	Without gastric dilatation
0.476–0.522	Suspicious
0.522–1.632	With gastric dilatation

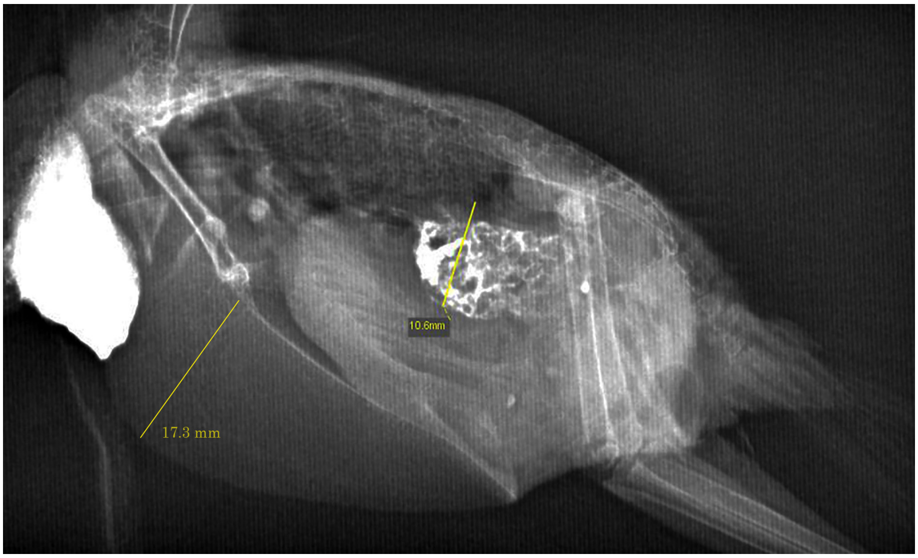

Figure 1. Total body radiographic image of lateral projection (Barium study). Ten min after the administration of barium sulfate (20 mL/kg, in the crop): The crop is dilated with contrast media. The proventriculus is dilated. Proventriculus diameter is 10.6 mm.

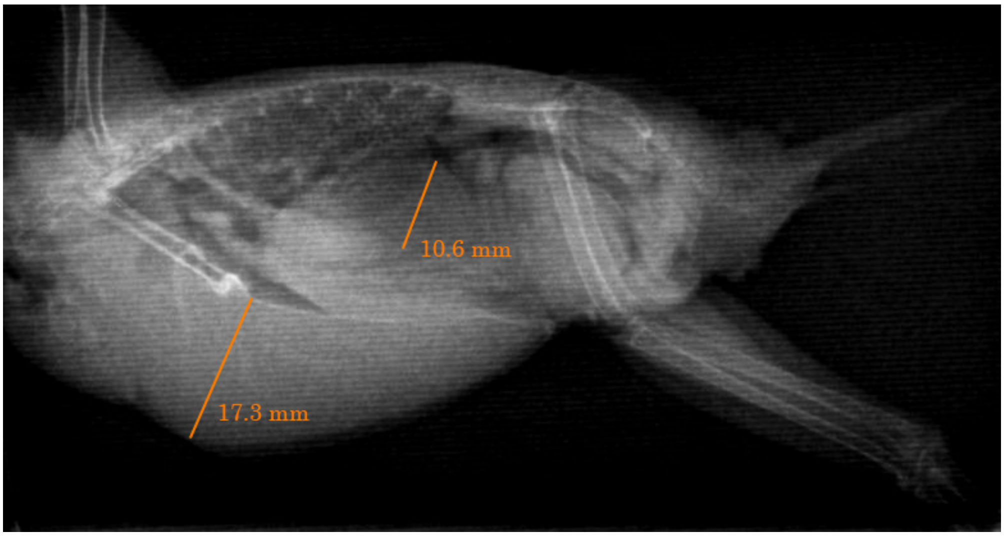

Figure 2. Total body radiographic image of lateral projection. The proventriculus is dilated. Proventriculus diameter is 10.6 mm.

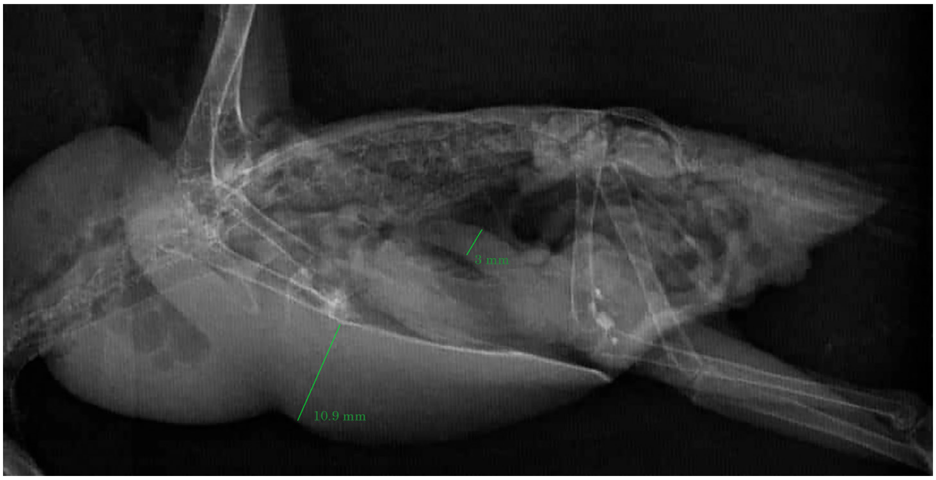

Figure 3. Total body radiographic image of lateral projection. Note the normal proventriculus diameter. The crop is distended with fluid material.

Table 2. Results of radiology and RT-PCR of birds are presented in the following table. -: negative; +: positive. In some cases, it was not possible to take radiographs from birds "-".

No.	Species	Sample	RT-PCR	Proventricular diameter-to-dorsoventral height of keel
1	Cockatiel	Feather	–	0.72
2	Cockatiel	Proventriculus	+	–
3	Cockatiel	Feather	–	0.54
4	Cockatiel	Feather	–	0.63
5	Cockatiel	Feather	+	0.30
6	Cockatiel	Proventriculus	+	–
7	Cockatiel	Proventriculus	–	–
8	Cockatiel	Proventriculus	–	–
9	Cockatiel	Feather	+	0.48
10	Cockatiel	Feather	+	0.43
11	Cockatiel	Feather	+	0.46
12	Cockatiel	Feather	+	0.34
13	Budgerigar	Feather	+	–
14	Budgerigar	Feather	+	–
15	Budgerigar	Feather	–	–
16	Budgerigar	Feather	+	–
17	Budgerigar	Feather	–	–
18	Budgerigar	Feather	–	–
19	Budgerigar	Feather	–	0.63
20	Budgerigar	Feather	–	0.76
21	Budgerigar	Feather	+	0.59
22	Budgerigar	Feather	–	0.65
23	Budgerigar	Feather	+	0.78
24	Budgerigar	Feather	+	0.57
25	Budgerigar	Feather	+	0.62
26	Budgerigar	Feather	+	0.67

Continued

27	Budgerigar	Feather	–	0.66
28	Budgerigar	Feather	–	0.60
29	Budgerigar	Feather	+	0.56
30	Budgerigar	Feather	–	0.55
31	Budgerigar	Feather	–	0.43
32	Budgerigar	Feather	+	–
33	Budgerigar	Feather	–	0.44
34	Budgerigar	Feather	+	0.60
35	Budgerigar	Feather	–	0.49
36	Budgerigar	Feather	–	0.50
37	Budgerigar	Feather	–	0.59
38	Budgerigar	Feather	–	0.55
39	Budgerigar	Feather	+	0.59
40	Budgerigar	Feather	–	0.59

samples (50%) tested positive. Positive Bornavirus cases (%) did not show any significant difference between the Cockatiels and Budgerigars (p = 0.37). There was no significant difference in the prevalence of ABV in RT-PCR between birds <2 months old and >2 months old (p = 0.7). Afterward, the results of the radiographs and RT-PCR were compared in the samples. It was observed that among the 17 positive feather samples in the RT-PCR assay, four birds had a $\frac{\text{proventriculus diameter}}{\text{height of keel}}$ ratio less than 0.476 and a bird was also in the suspicious range. These results show that although radiology is a valuable diagnostic method, it cannot be used for a definitive diagnosis without further studies.

Clinical evaluation of positive birds by RT-PCR revealed that 10 birds did not properly digest food and undigested food was seen in their droppings, and six birds had vomiting. In a bird, vomiting and undigested food in the droppings were common symptoms. There was no significant difference in the prevalence of vomiting (p = 0.58) and emaciation (p = 0.55) between positive and negative RT-PCR results. The presence of undigested seed in droppings was seen more commonly in birds with positive RT-PCR than that of with negative RT-PCR (p = 0.011). Also, in five birds, neurological symptoms were recorded in their examination. These symptoms were usually manifested as paralysis and seizures in the later stages of the disease. Two birds died after the onset of neurological symptoms, and in necropsy, proventriculus dilatation and brain hyperemia were observed in these birds. There was no significant difference in the prevalence of neurological symptoms between birds that were positive and negative by RT-PCR (p = 0.44). Two birds did not have any specific clinical symptoms, but their RT-PCR results were positive. It is noteworthy to mention that the parents of both these two birds died of PDD-like symptoms. These birds may be vertically infected.

4. Discussion

Avian-bornavirus was reported as a novel pathogen responsible for a debilitating disease in psittacines in Europe, North America, South Africa, Brazil, Japan, and Thailand [4–7,26,27]. To our knowledge, this is the first report addressing PDD cases in psittacines of Iran by radiology and RT-PCR. Ante-mortem diagnosis of PDD is often challenging and usually is done by histological assessment of crop biopsies [28]. In this regard, we ascertain the usefulness of two common diagnostic assays, namely radiology and RT-PCR, for the evaluation of referred birds with clinical suspicion of PDD. Gastrointestinal symptoms were more frequently observed in birds than neurological symptoms. Results of our study revealed that passing whole seed in the droppings is the most prevalent clinical symptom among birds with positive RT-PCR. In a study by Silva et al., a strong link was reported between body condition score and the presence of bornavirus in RT-PCR [29]. Their findings contrasted with the results of the present study which showed no significant difference in the prevalence of emaciation between negative and positive birds by RT-PCR. After a clinical examination, the birds were evaluated for PDD using radiology. According to the radiographs, 55.5% of the evaluated birds were considered positive. Although radiology can be a reliable diagnostic assay in PDD cases [30], large proventriculus may be seen in some healthy parrots or neonates [31]. Hellebuyck et al., described a proventriculus dilatation in a Macaw by radiographic examination. According to their findings, bacterial proventriculitis was the cause of this dilatation in radiography. The bird was negative for Parrot Bornavirus by RT-PCR [32]. Wyss et al., reported no significant difference in proventriculus diameter-to-keel height ratio between positive and negative macaws which were assorted by crop biopsy. They recommended radiographic evaluation of proventriculus diameter as a useful screening tool for the detection of PDD-suspected birds [33]. It can be concluded that radiographic examination of proventriculus provides evidence to support PDD diagnosis in birds. Although, this evidence should be confirmed by other diagnostic assays.

Molecular assays, such as RT-PCR, are employed by researchers for the diagnosis of PDD in birds. Sassa et al., investigated the prevalence of the ABV genome by RT-PCR among pet birds in Japan. They found that four out of 93 samples yielded positive in this assay. Positive birds for ABV-4 and ABV-2 had gastrointestinal dysfunction and behavioral problems, respectively [27]. Another study was undertaken by Philadelpho and coworkers to investigate the prevalence of ABV among 112 psittacine birds. According to their results, 28.6% of the samples were positive [8]. In 2019, Sa-Ardta and colleagues evaluated 111 psittacines of 22 different bird species for PDD by various diagnostic tests including RT-PCR, ELISA, and immunohistochemistry (IHC). Results of their study revealed that 54.1% of the samples were infected with the bornavirus [4]. Donatti et al., examined carcasses of 20 psittacines that were suspected of PDD and more analysis was done on 10 of those carcasses by histopathology, RT-PCR, and IHC for ABV. All the birds were found positive by histopathology. However, 40% of samples were confirmed for ABV in RT-PCR and IHC. The results of their study revealed that the efficiency of these assays is different, and it is necessary to consider the limitations of these assays for the detection of PDD patients [7]. Most of the birds in the present study had gastrointestinal disorders and neurological symptoms. Therefore, the prevalence rate of PDD in birds was reported high. We used RT-PCR and radiography for ante-mortem diagnosis of PDD and monitoring purposes of birds in North East of Iran. Feathers are noninvasive and easy-to-obtain samples that were suggested previously as reliable materials for RT-PCR of ABV [34]. Further analysis is underway for sequencing these strains and comparing them with worldwide strains. The results of this study revealed that the radiology of birds for PDD signs and molecular detection of the ABV genome are useful tools for the diagnosis of PDD in birds. It is recommended to study a larger population of submitted birds from different species to ascertain the prevalence of PDD among birds in this region. Furthermore, other tools are also included in the study for definitive diagnosis of the disease and comparing the efficacy of RT-PCR and radiography with them.

In conclusion, our results demonstrated that the prevalence of proventricular dilatation and the presence of ABV, respectively by radiology and RT-PCR, was high in clinically referred birds with gastrointestinal and neurological symptoms, and this finding indicates the need for further evaluation of all referred birds.

The biggest problem seems to occur when the density of ornamental birds increases and the hygiene and quarantine of new birds are not considered. Thus, the implementation of biosecurity rules and regular testing of birds is recommended to avoid the spread of ABV among psittacines.

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The authors declare no conflict of interest.

Informed consent
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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