Biomolecular Detection and Quantification xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Biomolecular Detection and Quantification

journal homepage: www.elsevier.com/locate/bdq

Short communication

Incidence and detection of beak and feather disease virus in psittacine birds in the UAE

F. Hakimuddin [a,*], F. Abidi [a], O. Jafer [a], C. Li [a], U. Wernery [b], Ch. Hebel [c], K. Khazanehdari [a]

[a] Molecular Biology and Genetics Laboratory, P.O. Box 597, Dubai, United Arab Emirates
[b] Central Veterinary Research Laboratory, P.O. Box 597, Dubai, United Arab Emirates
[c] German Veterinary Clinic, P.O. Box 34867, Abu Dhabi, United Arab Emirates

ARTICLE INFO

Article history:
Received 31 May 2015
Received in revised form 27 August 2015
Accepted 7 October 2015
Available online xxx

Keywords:
Psittacine beak and feather disease (PBFD) virus
Real-time PCR
Psittacine birds
Circovirus
Sequencing

ABSTRACT

Beak and feather disease is caused by Circovirus, which affects actively growing beak and feather cells of avian species. The disease affects mainly young birds while older birds may overcome the disease with few lasting effects. Due to lack of treatment, the only way to control the disease is through hygiene and early diagnosis. As a diagnostic tool, we have established a Taqman probe based real-time PCR assay to detect the presence of the viral genome in psittacine birds in UAE and reported the incidence of circovirus in different species of psittacine birds. The sensitivity of our assay was found to be very high with detection limit of up to 3.5 fg of DNA in the sample. The mean prevalence of circovirus was found to be 58.33% in African Grey Parrots, 34.42% in Cockatoos, 31.8% in amazon parrots and 25.53% in Macaws.

The Taqman assay is a quick, reliable and sensitive detection method that has been instrumental in identifying this disease that was not previously reported in the region.

1. Introduction

Psittacine beak and feather disease (PBFD) is mostly observed in Australian and South African avian species [5]. PBFD was first recognized and described thoroughly in 1975 by Dr. Ross Perry, a veterinary practitioner in Sydney. A number of birds showing feather and beak abnormalities were identified, initially in private collections and in wild flocks in Australia, but subsequently spreading rapidly around different parts of the world (http://www.environment.gov.au).

PBFD has since then been recognized as one of the most significant diseases of psittacine birds worldwide. Initial theories as to its cause included genetic or deficiency problems. Research at Murdoch University, Australia, and the University of Georgia, USA has demonstrated its cause to be an extremely small circovirus, which is a small, non-enveloped DNA virus of the Circoviridae family. It has a single-stranded, circular-DNA genome [12,17] that is 16 nm in diameter, 1993 nucleotides in length, encodes three proteins and is surrounded by a spherical capsid with icosahedral symmetry. The virus specifically infects psittacine birds and is mostly fatal in young birds. Cockatoos, Macaws, African Grey Parrots and Ring-necked parakeets are some of the species known to be susceptible to this virus. Besides Psittaciformes, the virus also infects other avian families like Columbiformes, Passeriformes and Anseriformes [13]. Circovirus, typically, targets actively growing cells in beak, claws and feather follicles causing feather malformation and feather loss. Besides that, it affects the bursa fabric and the thymus, causing immunosuppression, as lymphocyte production becomes limited.

Although, the virus is known to occur naturally in the wild population, the distribution of the disease and factors involved in its spread are not well understood. The virus spreads horizontally – to adjacent birds by direct contact; and in adult carrier birds that reach breeding age, vertically – through the eggs and chicks affecting the next generation. Virus infectivity probably persists in contaminated nests for many months or even years (http://www.theparrotsocietyuk.org).

Young birds usually succumb to the infection while older birds may overcome the disease with few lasting effects. Surviving birds are known to shed the virus and a small percentage of birds acquire lasting immunity [9]. Whilst many attempts have been made to produce a vaccine to combat the infection, to date none has been successful. There is no known treatment and the only way to control the disease is through hygiene, strict isolation or culling of all infected birds. This stipulates the importance of early diagnosis of

* Corresponding author. Tel.: +971 4 3372529; fax: +971 4 3372769.
E-mail address: fhakim@mbg.ae (F. Hakimuddin).

http://dx.doi.org/10.1016/j.bdq.2015.10.001

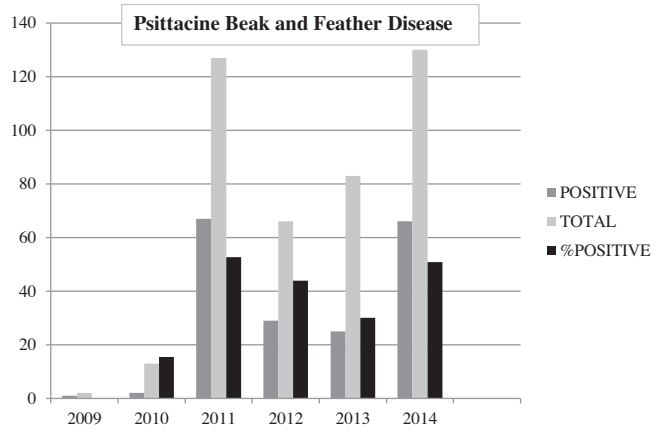

Fig. 1. Year wise (2009–2014) distribution of the total number of samples tested for Circovirus and number of samples identified as positive by real-time PCR.

the disease to control its progression in infected birds and avoid its spread amongst uninfected ones.

Psittacine beak and feather disease has been found to be widespread in different countries of the world with a reported prevalence of 23% in Australia [5], 40.4% in Germany [10], 8% in Italy [1], 41.2% in Taiwan [3] and 3.5–4% in USA [2]. The disease has been identified previously using traditional serological methods. Raidal et al. [11] have reported anti-PBFD antibodies in Australian psittacine species and Macwhirter [7] has reported the presence of antibodies against the virus in the range of 41–94% in sulfur crested Cockatoos, galahs, little corellas and long billed corellas. However, serological tests are good indicators of immune status and presence of chronic disease in the bird, but are of little value in predicting a clinical diagnosis of the disease. More recently, molecular based tests [4,8,15,18] that use specific primers for the target sequence, have been more successful in making a timely diagnosis of the disease.

In the past few years, there has been an increase in the international trade of live birds across the world [7]. The bird markets in the UAE are also swamped with birds imported from different countries that may be carriers of diseases. These birds are usually bought by owners from these markets and housed as pets or held captive in private farms.

We report here for the first time in the UAE, the prevalence of circovirus causing beak and feather disease, through detection of viral particles in blood and feathers of psittacine birds using a highly sensitive Taqman probe-based real-time PCR assay that is established in our laboratory. As the PCR method detects the virus (antigen), the assay can detect active as well as latent infection.

2. Materials and methods

A total of 421 blood or feather samples from pet shops, veterinary clinics, private owners and wildlife parks were received for testing over a period of five years, from October 2009 to December 2014. Although we recommended and mostly received blood for testing, some clients submitted feather samples instead. Fig. 1 shows the number of samples received each year and the number of positive samples identified. The species tested included African Grey Parrots, Macaws, Cockatoos, Parakeets, Galahs, Amazon and Conure.

2.1. Extraction of DNA from blood

DNA was extracted from EDTA blood using the Qiagen DNeasy blood and tissue kit (Qiagen Ltd., Crawley, United Kingdom) according to the manufacturer's instructions. Briefly, 10 μL of blood was mixed with 190 μL of PBS and 20 μL of proteinase K was added. After addition of 200 μL Buffer AL, the sample was vortexed and incubated at 56 °C for 10 min. Then, 200 μL ethanol (96–100%) was mixed with the sample and it was loaded into a DNeasy Mini Spin Column for purification and DNA was eluted in 50 μL of elution buffer and stored at 4 °C. DNA was quantified using Nanodrop ND-1000 spectrophotometer (USA).

2.2. Extraction of DNA from feathers

Alternatively, DNA from roots of feathers was extracted using alkaline lysis (heating in 20 μL of 0.2 M NaOH for 7 min at 95 °C and neutralizing with 75 μL of 0.1 M Tris–HCl) and purified using phenol–chloroform method.

2.3. PCR amplification

Primers PBFD-F GCCCACGTGACTTCAAGACT and PBFD-R ACGGAGCATTTCGCAATAAG (Metabion, Germany) were designed using Primer 3 express software to amplify a 194-bp region of the replication associated protein gene (V1) (Gene bank accession DQ397817.1) of beak and feather disease virus isolate AFG5-ZA. These were used in conjunction with the Taqman probe Fam-TCGTGGGGACCTCGATCTCACTCG-Tamra. The real-time PCR was performed using Roche Light Cycler 2.0 (Manheim, Germany) under the following conditions: initial denaturation at 95 °C for 8 min, amplification at 95 °C for 10 s, 51 °C for 20 s and 72 °C for 30 s for 45 cycles. A Circovirus positive amplification control and negative control without DNA was included in the PCR. Samples that showed a Ct value of ≤40 and an exponential fluorescence were scored as positive and samples that did not fulfill these criteria were scored as negative.

2.4. Duplex PCR with internal control

To ensure the success of our extraction protocol, we incorporated an internal DNA extraction control (DEC) (Bioline, UK) in the sample. 4 μL of DEC was added to the sample or PBS (negative extraction control) at the lysis stage and extraction was done as above using the Qiagen DNeasy blood and tissue kit. Duplex PCR with internal control primer (control mix) (Bioline, UK) was setup with the same cycling conditions as above.

2.5. Sensitivity

Sensitivity of the test was determined by making 10-fold serial dilutions of DNA obtained from positive blood and feather samples with an initial concentration of 86 and 35 ng/μL, respectively. PCR amplification was performed using at least 8 log dilutions and a standard curve was prepared.

2.6. Sequencing

Circovirus positive DNA from 7 African Grey Parrots, 3 Macaws and 2 Cockatoos were selected for sequencing. Forward and Reverse primers were: PBFD-F and PBFD-R. Sequencing was done using dideoxy Sanger method and run on the ABI 3730 XL (USA). Sequences were analyzed using sequencing analysis software (Version 5.1.1) and aligned using DNAMAN.

Please cite this article in press as: F. Hakimuddin, et al., Incidence and detection of beak and feather disease virus in psittacine birds in the UAE, Biomol Detect Quantif (2015), http://dx.doi.org/10.1016/j.bdq.2015.10.001

2.7. Statistical analysis

T-test was done on the total number of captive and imported birds to see if there was a significant difference in the percentage of positive samples among captive and imported birds.

3. Results

Overall, 421 samples from eight different species of psittacine birds were tested for the presence of Circovirus using real-time PCR method. A total of 190 samples (45.13%) were found to be positive (Table 1) of which the year wise percentage in 2010, 2011, 2012, 2013 and 2014 were 15%, 53%, 44%, 30% and 51%, respectively (Fig. 1).

Circovirus was detected in a large number of African Grey parrots and the percentage of positive samples was found to be 58.33% whereas in Cockatoos about 34.42% of the samples tested were positive (Table 1). Macaws and Parakeets showed a good number of positives as well, with 25.53% samples being positive in Macaws and 16.66% of Parakeet samples detected as positive. Parrots of known and unknown species showed a positive percentage of 35.2% and comprised of Indian ring neck parrots, yellow parrots, Senegal parrots and others. Not all samples submitted to the lab had species identified (Table 1). The birds were also segregated into two groups based on where they came from. All those birds that belonged to the farms and wildlife centers were considered as captive while the birds that came from private owners, veterinary clinics and pet-shops were grouped as imported. In 2011, 43 out of a total of 69 captive birds were tested positive (Table 2). Total captive birds tested in 2012 and 2013 were 21 and 19 and the number tested positive were 8 and 5, respectively (Table 2). The total number of imported birds tested positive from 2010 to 2013 ranged from 2 to 20. The highest number of imported birds was tested in 2014, where 66 out of a total of 128 birds were positive for circovirus (Table 2). Figs. 2 and 3(a) show the amplification plots for a range of dilutions of circovirus positive DNA from blood and feather carried out to determine sensitivity of the test. DNA from blood (initial concentration 86 ng/μL) and feather (initial concentration 35 ng/μL) was serially diluted 10 fold. Ct values for the first and last dilutions were 15 and 37 for blood and 18 and 39 for feather, respectively. Results indicate that Circovirus was detected from as low as 8.5 fg of total DNA (Fig. 2(a)) and 3.5 fg of total DNA (Fig. 3(a)) from blood and feather samples, respectively. Standard curves obtained for circovirus DNA from blood and feather are shown in Figs. 2 and 3(b). The PCR efficiency values for each of these was 1.9 and 2.1 respectively.

Fig. 2(c) depicts corresponding amplification curves obtained for the internal control in the 560 channel. The first dilution having the lowest Ct value in the target 530 channel shows suppression of IC amplification as would be expected for a strongly positive sample.

Fig. 4 shows multiple sequence alignment of circovirus DNA from seven African Grey Parrots, three Macaws and two Cockatoos compared to an NCBI reference sequence (gi: 90994631). The results show some sequence variations in circovirus sequences of three African Grey Parrots (AGP2, AGP3 and AGP4) compared to the virus sequence from other species.

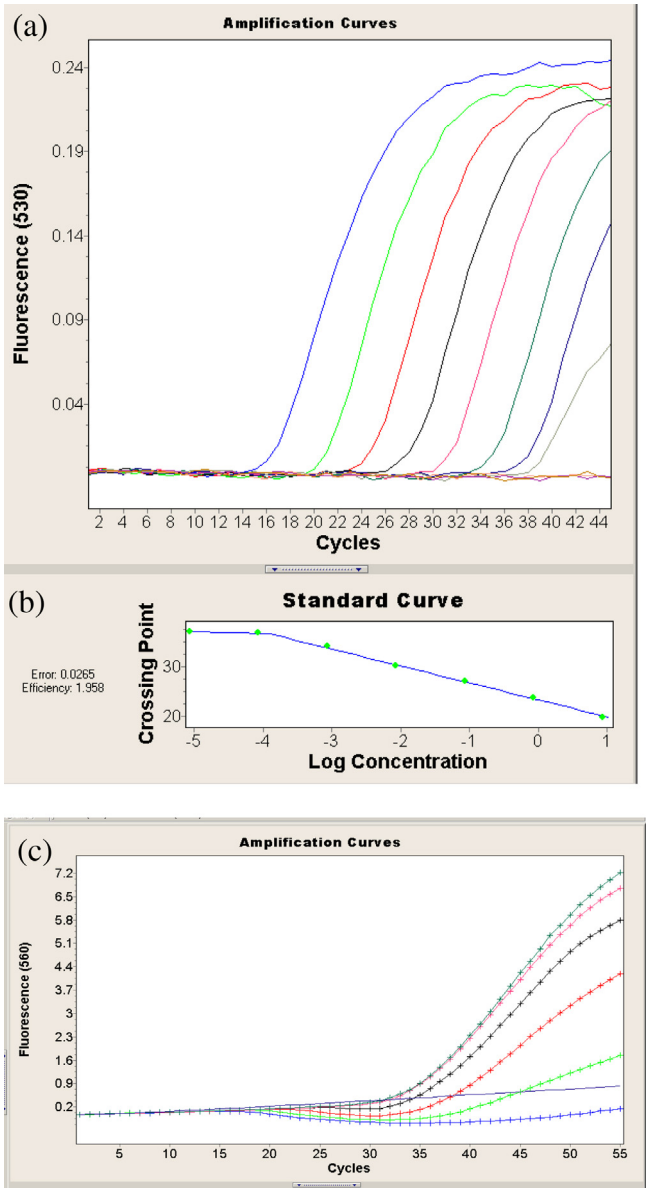

Fig. 2. (a) Amplification plot of circovirus positive DNA from a blood sample serially diluted to 8 log dilutions with an initial concentration of 86 ng/μL. (b) Standard curve of log10 ng DNA concentration *vs* Ct value obtained from a circovirus positive blood sample. (c) Amplification plot of internal extraction control (DEC) with 1:10 serial dilutions of circovirus positive blood sample.

4. Discussion

PBFD is a debilitating disease of psittacine birds and may have serious implications such as huge economic loss or cross species transmission of the disease. Also, non-availability of viable treatment options may lead to endangering of the species. Until recently, the disease was only identified when clinical signs like deformed beak and feathers appeared on the bird. Serological tests such as heamagglutination (HA), heamagglutination inhibition (HI) and ELISA have been inconsistent due to unavailability of standardized reagents [11,16]. Culturing the virus *in vitro* has also proven to be difficult [14]. Therefore, accurate and timely diagnosis of the disease using rapid and sensitive methods is imperative, to track this disease in the region and implement effective control measures.

In 2009, we established a real-time Taqman PCR assay in our Laboratory, to detect psittacine beak and feather disease virus (circovirus). The assay is very sensitive, specific and reliable and can detect viral particles from as low as 3.5 fg of total DNA from the sample. In the first year (2009) one bird sample submitted by a farm owner tested positive using this assay. Since then, there has been a constant rise each year in the number of samples submitted for Circovirus testing (Fig. 1). From the high number of African Grey Parrots, Macaws and Cockatoos submitted for analysis, it seems that these birds are more popular with private owners (Table 1). This is

Please cite this article in press as: F. Hakimuddin, et al., Incidence and detection of beak and feather disease virus in psittacine birds in the UAE, Biomol Detect Quantif (2015), http://dx.doi.org/10.1016/j.bdq.2015.10.001

Table 1
Real-time PCR results of beak and feather disease virus in different psittacine species of UAE tested between 2009 and 2014.

Species	Scientific name	Total	Positive (%)
African grey parrot	(*Psittacus erithacus*)	216	126(58.33)
Parakeet	(*Melopsittacus undulates*)	18	3(16.66)
Cockatoo	(*Cacatuoidea*)	61	21(34.42)
Amazon parrot	(*Amazona amazonica*)	22	7(31.8)
Conure	(*Aratinga solstitialis*/*Pyrrhura molinae*)	1	–
Macaw	(*Ara ararauna*)	47	12(25.53)
Galah	(*Eolophus roseicapillus*)	2	2
Parrot (unknown species)		54	19(35.2)
Total		421	190(45.13)

Table 2
Year wise (2009–2014) distribution of the number of samples from captive and imported birds that were tested for Circovirus using real-time PCR assay (% was not calculated for birds <5 in a group; *p*-value according to *T*-test was calculated to be 0.15).

Captive *vs* imported	Pos captive	Pos imported	Total captive	Total imported	% Pos captive	% Pos imported
2009	1	0	1	1	–	–
2010	0	2	4	9	–	22.22
2011	43	24	69	58	62.32	41.4
2012	8	21	21	45	38	46.66
2013	5	20	19	64	26.31	31.25
2014	0	66	2	128	–	51.56
Total	57	133	116	305	49.13	43.6

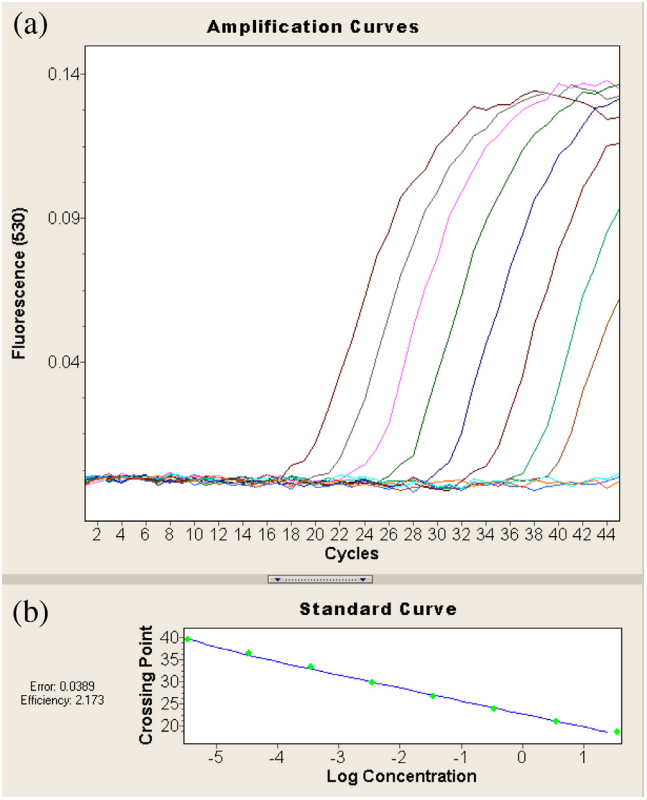

Fig. 3. (a) Amplification plot of circovirus positive DNA from a feather sample serially diluted to 8 log dilutions with an initial concentration of 35 ng/μL. (b) Standard curve of $\log_{10}$ ng DNA concentration *vs* Ct value obtained from a circovirus positive feather sample.

the first report on the incidence and detection of PBFD virus in the UAE. Comparative studies with other methods were not done. The study was also limited by the type and number of samples and the background information provided to us by clients.

The findings of the study indicate that the mean prevalence of circovirus was quite high with 190 samples identified as positive from a total of 421 samples. Among the different species tested, African Grey Parrots (58.33%) showed high prevalence followed by Cockatoos (34.42%), Macaws (25.53%) and Parakeets (16.6%). The high prevalence in African Grey Parrots could be due to the large number of samples of these birds submitted for analysis (Table 1). Unfortunately, tracing the origin of the infected birds and the origin of the infection proved to be difficult. It was therefore not possible to indicate if the birds were infected on arrival or got infected in the bird markets where hygiene standards are questionable.

Captive breeding programs carried out in Australian wild species of parrots have shown that the disease has the potential to spread rapidly among captive birds (http://www.environment.gov.au). Based on our categorization of imported and captive birds, we tried to see if this holds true with our results. In 2011, a high proportion of captive birds (69 birds) were tested, of which 43 (41.4%) were found to be positive for circovirus (Table 2). Whether these birds get infected with the newly added imported birds that are carriers of the disease or whether they are naturally infected is not known. Though an assumption can be made in concurrence with the Australian study that this high positivity may be due to the rapid spread of infection in the farm, it needs to be supported by more such observations and data on captive birds. Unfortunately, enough samples were not received from captive birds during the other years to get significant ratios.

The number of imported birds received for testing seemed to steadily increase over the years with the highest number (128 birds) being tested in 2014. Of these, 66 (51.56%) birds were positive. The other years also showed a good number of circovirus positive birds.

Combined statistical results (*T*-test) suggest that there is no significant difference in the incidence of circovirus between imported and captive birds (Table 2). This, however, could be affected by lack of clear differentiation of the two groups.

Sequencing of circovirus isolates from three different psittacine species was performed to see if there were any significant differences in the PBFD virus that could point to the high positive

Please cite this article in press as: F. Hakimuddin, et al., Incidence and detection of beak and feather disease virus in psittacine birds in the UAE, Biomol Detect Quantif (2015), http://dx.doi.org/10.1016/j.bdq.2015.10.001

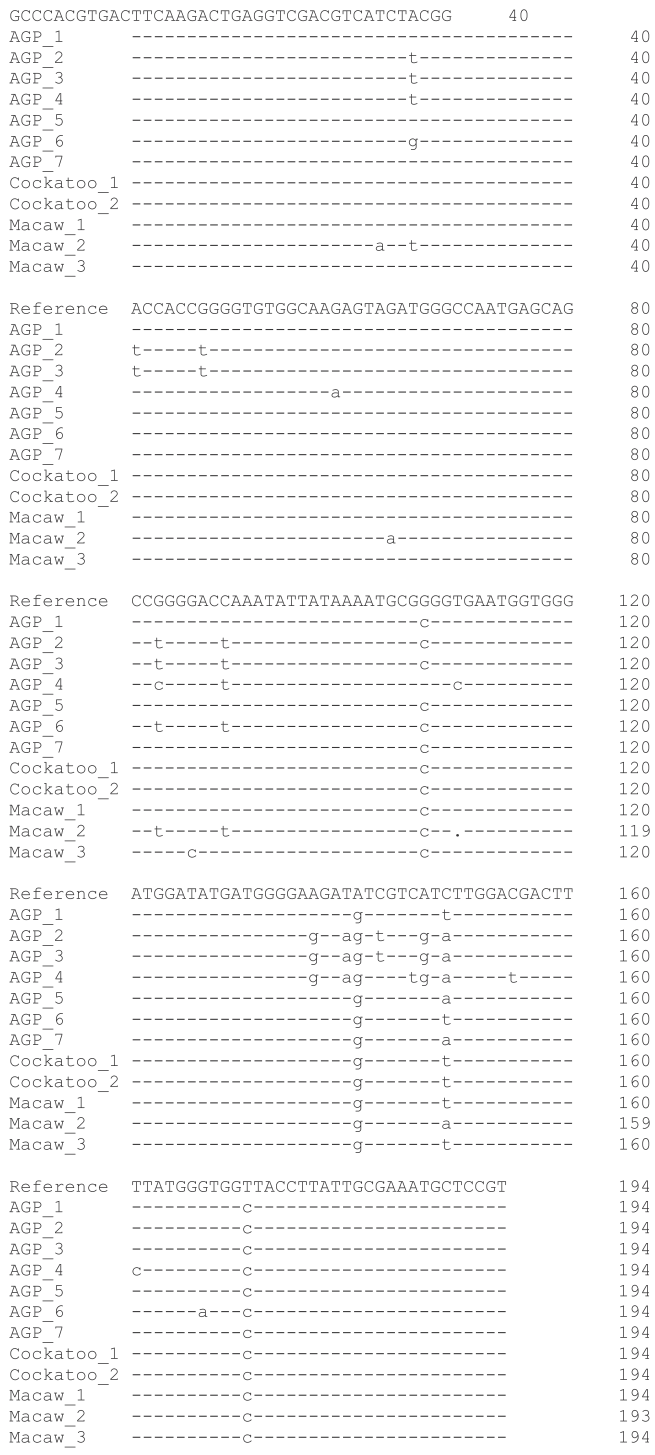

Fig. 4. Multiple sequence alignment of PBFD virus sequences from 7 African Grey Parrots (AGP), 3 Macaws and 2 Cockatoos compared to an NCBI reference sequence (gi: 90994631).

percentage observed in African Grey Parrots. Twelve PBFD virus from positive samples (seven African Grey Parrots, two Cockatoos and three Macaws) was randomly selected for sequencing. Fig. 4 shows the multiple sequence alignment of these sequences compared to a reference sequence (gi: 90994631). Although the sequenced fragment is short, it can be noticed that there are distinct sequence variation of the PBFD virus sequence obtained from the three African Grey Parrots (AGP2, AGP3 and AGP4) (Fig. 4) compared to the virus sequence obtained from the other species. As the viral sequence obtained from the other four African Grey Parrots is similar to the sequences obtained from Macaw and Cockatoo samples, the difference seen may be attributed to the origin of the virus and not the species. More work needs to be done to elucidate this.

The study warrants thorough screening of all birds before import and only circovirus free birds should be allowed to enter the UAE with extra vigilance on birds coming from countries where the disease is endemic.

It is worth mentioning that it is imperative not only to test psittacine birds for PBFD when they are sick, but also when they are purchased. Awareness about the severity and spread of the disease should also be created among private owners and farm managers so they can manage small outbreaks. A more extensive surveillance approach should be adopted to prevent any major outbreak of this debilitating disease in the future. Also, as a future goal, additional screening needs to be done in species other than psittacine birds to provide conclusive results about the actual prevalence of the disease in the region.

Acknowledgements

The authors would like to thank Dr. Ali Ridha, administrative director of CVRL, Dubai, UAE, for his continuous support. The authors are grateful to Dr. Monika Rinder, Ludwig-Maximilian-University, Munich, for providing the initial positive control. This study was financially supported by H. H. Sheikh Mohammed Bin Rashid Al Maktoum, Vice President and Prime Minister of UAE and Ruler of Dubai.

References

[1] E. Bert, L. Tomassone, C. Peccati, M.G. Navarrete, S.C. Sola, Detection of beak and feather disease virus (BFDV) and avian polyomavirus (APV) DNA in psittacine birds in Italy, J. Vet. Med. B Infect. Dis. Vet. Public Health 52 (2005) 64–68.
[2] E. de Kloet, S.R. de Kloet, Analysis of the beak and feather disease viral genome indicates the existence of several genotypes which have a complex psittacine host specificity, Arch. Virol. 149 (2004) 2393–2412.
[3] C.M. Hsu, C.Y. Ko, H.J. Tsaia, Detection and sequence analysis of avian polyomavirus and psittacine beak and feather disease virus from psittacine birds in Taiwan, Avian Dis. 50 (2006) 348–353.
[4] H. Katoh, K. Ohya, H. Fukushi, Development of novel real-time PCR assays for detecting DNA virus infections in psittaciform birds, J. Virol. Methods 154 (2008) 92–98.
[5] B. Khalesi, N. Bonne, M. Stewart, M. Sharp, S.A. Raidal, Comparison of haemagglutination, haemagglutination inhibition and PCR for the detection of psittacine beak and feather disease virus infection and a comparison of isolates obtained from loriids, J. Gen. Virol. 86 (2005) 3039–3046.
[7] P. Macwhirter, Viral diseases of concern, in: Birds 2000. Proceedings Number 334 of the Post., Graduate Foundation in Veterinary Science, University of Sydney, Sydney, 2000, pp. 171–184.
[8] H. Ogawa, T. Yamaguchi, H. Fukushi, Duplex shuttle PCR for differential diagnosis of budgerigar fledgeling disease and psittacine beak and feather disease, Microbiol. Immunol. 49 (2005) 227–237.
[9] D.A. Pass, R.A. Perry, The pathology of psittacine beak and feather disease, Aust. Vet. J. 61 (1984) 69–74.
[10] M. Rahaus, M.H. Wolff, Psittacine beak and feather disease: a first survey of the distribution of beak and feather disease virus inside the population of captive psittacine birds in Germany, J. Vet. Med. B Infect. Dis. Vet. Public Health 50 (2003) 368–371.
[11] S.R. Raidal, M. Sabine, G.M. Cross, Laboratory diagnosis of psittacine beak and feather disease by haemagglutination and haemagglutination inhibition, Aust. Vet. J. 70 (1993) 133–137.
[12] B.W. Ritchie, F.D. Niagro, P.D. Lukert, K.S. Latimer, W.L. Steffens, N. Pritchard, A review of psittacine beak and feather disease: characteristics of the PBFD virus, J. Assoc. Av. Vet. 3 (1989) 143–149.
[13] B.W. Ritchie, P.D. Lukert, Psittacine beak and feather disease, in: A Laboratory Manual for the Isolation, Identification and Characterization of Avian Pathogens, fifth ed., American Association of Avian Pathologists, Athens, 2008, pp. 122–123.
[14] P. Shearer, Development of novel diagnostic and vaccine options for beak and feather disease virus (BFDV), in: PhD Thesis, Murdoch University, 2008.
[15] P.L. Shearer, M. Sharp, N. Bonne, P. Clark, S.R. Raidal, A quantitative real time PCR assay for beak and feather disease virus, J. Virol. Methods 159 (2009) 98–104.

Please cite this article in press as: F. Hakimuddin, et al., Incidence and detection of beak and feather disease virus in psittacine birds in the UAE, Biomol Detect Quantif (2015), http://dx.doi.org/10.1016/j.bdq.2015.10.001

[16] P.L. Shearer, M. Sharp, N. Bonne, P. Clark, S.R. Raidal, A blocking ELISA for the detection of antibodies to psittacine beak and feather disease virus, J. Virol. Methods 158 (2009) 136–140.
[17] D. Todd, F.D. Niagro, B.W. Ritchie, W. Curran, G.M. Allan, P.D. Lukert, K.S. Latimer, W.L. Steffens, M.S. McNulty III, Comparison of three animal viruses with circular single-stranded DNA genomes, Arch. Virol. 117 (1991) 129–135.
[18] Ypelaar, A universal PCR for detection of psittacine beak and feather disease virus, Vet. Microbiol. 68 (1999) 141–148.

Further reading

http://www.theparrotsocietyuk.org/veterinary-advice/psittacine-beak-and-feather-disease.http://www.environment.gov.au/system/files/resources/a13239ba-fb01-4c31-9fa8-519dcbc593ca/files/p-c-disease.pdf.

Please cite this article in press as: F. Hakimuddin, et al., Incidence and detection of beak and feather disease virus in psittacine birds in the UAE, Biomol Detect Quantif (2015), http://dx.doi.org/10.1016/j.bdq.2015.10.001