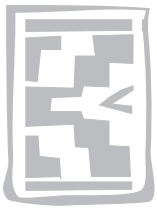

Psittacine beak and feather disease virus in budgerigars and ring-neck parakeets in South Africa

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ABSTRACT

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Psittacine beak and feather disease (PBFD) is a common disease of the psittacine species and is caused by the psittacine beak and feather disease virus (PBFDV). In this study the occurrence of the disease in ring-neck parakeets and budgerigars in South Africa suffering from feathering problems, using polymerase chain reaction as a diagnostic test was investigated. The genetic variation between viral isolates was also studied. Results indicate that PBFDV can be attributed to being the cause of feathering problems in some of the ring-neck parakeets and budgerigars in South Africa. Genetic variation of isolates occurs between species and individuals. A cheap and easy to use method of blood sample collection on filter paper for diagnostic purposes was also evaluated. It proved to be less stressful to the birds and did not inhibit further processes.

Keywords: Budgerigars, genetic variation, psittacine beak and feather disease virus, ring-neck parakeets, South Africa

INTRODUCTION

Psittacine beak and feather disease (PBFD) is a viral disease that was first discovered in 1975 in cockatoos in Australia. It is caused by the psittacine beak and feather disease virus (PBFDV) which is a small (14–18 nm diameter), icosahedral, non-enveloped virus with a circular single-stranded DNA (ssDNA) genome that has been characterised and placed in the family *Circoviridae* (Raidal, Firth & Cross 1993; Studdert 1993; Pass, Plant & Sexton 1994; Sexton, Penhale, Plant & Pass 1994; Ramis, Latimer, Gibert & Campagnoli 1998; Ypelaar, Bassami, Wilcox & Raidal 1999; Todd 2000).

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The disease occurs predominantly in sulphur-crested cockatoos (*Cacatua galerita*) but also in budgerigars (*Melopsittacus undulatus*) and other psittacine birds. Characteristic clinical signs include symmetric feather dystrophy and loss and deformity of the beak in some species (Ramis *et al*. 1998). Birds infected with PBFDV usually die as a result of secondary infections, which support the view that they are immunosuppressed; bursal and thymic lesions are consistently detected (Todd 2000).

Histopathology has been used for the routine diagnosis of PBFD, but more recently, polymerase chain reaction (PCR) and haemagglutination (HA) tests have been described for the detection of PBFDV and haemagglutination inhibition (HI) for the detection of antibodies to PBFDV (Riddoch, Raidal, & Cross 1996). Since all attempts to cultivate PBFDV

have so far been unsuccessful, the diagnosis of the disease must be confirmed by the demonstration of viral antigen or viral nucleic acid (Todd 2000).

Bassami, Ypelaar, Berryman, Wilcox & Raidal (2001) studied the genetic diversity of PBFDV and concluded that there was no evidence that distinctly different genotypes occur although various differences due to small deletions or insertions are present in the genomes of the isolates. They stated that the significance of these differences is unknown but that there is little evidence to support a relationship between genetic variation and regional distribution of isolates, or that there are differences in pathogenicity, antigenicity or any physiochemical characteristics of the virus.

There is no known cure for PBFD but a vaccine consisting of inactivated PBFDV in a double-oil emulsion adjuvant system (DOE vaccine) has been investigated (Raidal *et al*. 1993; Raidal & Cross 1994), and its use can be a safe and effective aid for controlling the disease if it is combined with other management procedures, such as good biosecurity.

In the disease, avian polyoma, caused by the avian polyomavirus (APV), clinical and pathological signs similar to those of PBFD are manifested. It has been known to occur in concurrent outbreaks with PBFD although documentation of these occurrences is limited (Latimer, Niagro, Campagnoli, Ritchie, Pesti & Steffens 1993; Ramis *et al*. 1998). Differentiation between the two diseases is difficult as the macro- and microscopical pathology of the two diseases is similar; diagnosis of avian polyoma disease is performed using techniques such as DNA *in situ* hybridization or PCR (Ramis *et al*. 1998).

Bird breeders in South Africa have been reporting feathering problems in budgerigars and have suspected that this may be due to APV infection. However, Bragg (2002) reported that PBFDV is widespread in parrots of South Africa breeders and suggested that the feathering problems in the budgerigars could be due to PBFDV infection and not APV infection.

This study was undertaken to determine if the occurrence of PBFD in budgerigars and ring-neck parakeets in South Africa exists using PCR as an identification tool. It was also undertaken to determine if there is any genetic variation between the isolates. A simple method for collecting blood samples on filter paper was also evaluated for its use in downstream processes for example the extraction of viral DNA followed by PCR.

MATERIALS AND METHODS

A total of seventeen birds were obtained from breeders in South Africa that showed feathering problems indicative of PBFDV infection. Blood samples were collected in heparin lithium tubes from six ring-neck parakeets in Bloemfontein, five budgerigars in Pretoria and five in Cradock, South Africa (Table 1). In addition, the effectiveness of blood sample collection on filter paper was evaluated. A strip of Whatman No.1 filter paper of approximately 5 mm in width and 40 mm in length was sterilized by U.V. radiation. A wing vein of one ring-neck parakeet (P-BLM-4) was pricked with a sterile 0.33 mm diameter needle and four separate strips of filter paper was used to absorb blood resulting in the coverage of a blood-soaked area of approximately 5 x 5 mm in size at one end of the filter paper. The filter papers were air-dried vertically at room temperature followed by viral DNA extraction from the samples after 24, 48, 72 and 96 h. In addition, a blood spot sample from a ring-neck parakeet in George, South Africa (P-GEO) that was submitted on a commercially available specimen collection paper was similarly processed after one month of storage at –20 °C.

Viral DNA was extracted from the blood samples using the QIAamp® DNA Blood Mini Kit and dried blood spot samples were processed using the QIAamp® DNA Mini Kit according to the manufacturer's instructions (Qiagen). The PCR amplifica-

TABLE 1 Blood samples obtained from species and sources

Sample	Species	Source
P-BLM-1	Parakeet	Bloemfontein
P-BLM-2	Parakeet	Bloemfontein
P-BLM-3	Parakeet	Bloemfontein
P-BLM-4	Parakeet	Bloemfontein
P-BLM-5	Parakeet	Bloemfontein
P-BLM-6	Parakeet	Bloemfontein
P-GEO	Parakeet	George
B-PTA-1	Budgerigar	Pretoria
B-PTA-2	Budgerigar	Pretoria
B-PTA-3	Budgerigar	Pretoria
B-PTA-4	Budgerigar	Pretoria
B-PTA-5	Budgerigar	Pretoria
B-CRA-1	Budgerigar	Cradock
B-CRA-2	Budgerigar	Cradock
B-CRA-3	Budgerigar	Cradock
B-CRA-4	Budgerigar	Cradock
B-CRA-5	Budgerigar	Cradock

tion was performed using a set of oligonucleotide primers [PB F1 (AACCCTACAGACGGCGAG-3') and PB R1 (5'-GTCACAGTCCTCCTTGTACC-3')] obtained from literature (Ypelaar *et al*. 1999; Bassami *et al*. 2001), amplifying a region of open reading frame 1 with a predicted size of 717 bp. Amplification was carried out according to Ypelaar *et al*. (1999) with a slight modification as follows; denaturation was carried out at 94 °C and annealing was performed at 55 °C. A second set of primers [PB F2 (5'-AACCATGCCGTCCAAGGA-3') and PB R2 (5'-TATCAGTAATTGATGGGGTGGG-3')] was designed to amplify the complete region of Open Reading Frame 1 of the PBFDV genome (GENBANK, Accession number AF080560) resulting in an amplicon with a predicted size of 876 bp.

Amplified products were identified by electrophoresis on 0.8 % agarose gels and visualized under UV illumination. Both primer sets produced amplicons of the expected size and those obtained with the primer set PB F1 and PB R1 were used in subsequent reactions. Restriction digests were performed to determine if there was any genetic difference between the isolates by means of digestion with the restriction endonuclease *Hae*III. The digest products were identified by electrophoresis on 2.5% low melting point agarose gels and visualized under UV illumination together with a 50 bp step ladder (Promega) as a molecular weight marker. Due to the possibility of different amplified products from samples B-CRA-1, B-CRA-2, B-CRA-4 and B-CRA-5, these amplicons were cloned into pGEM™ TEasy Vector System (Promega), small scale plasmid isolation were performed, the inserts removed from two randomly selected clones and digested with *Hae*III. The products were again visualized by electrophoresis.

RESULTS

All samples obtained from the ring-neck parakeets and budgerigars (except for sample B-CRA-3) produced PCR amplicons of the expected size of approximately 700 bp, using primer set PB F1 and PB R1. Sample B-CRA-3, that consistently produced a negative result, was further used as a negative control The positive samples amplified with primer set PB F2 and PB R2 also produced fragments of the expected size of approximately 850 bp (data not shown).

Amplicons obtained using primer set PB F1 and PB R1 were submitted to restriction digestion using *Hae*III. The digest profile was similar for all the ring-neck parakeet samples obtained from Bloemfontein with approximate sizes of 450 bp, 250 bp and a third smaller fragment of approximately 50 bp (Fig. 1, lanes 2–7). The profile for sample P-GEO differed in size from the other ring-neck parakeet samples yielding fragments of approximate sizes 460 bp, 250 bp and less than 50 bp (Fig. 1, lane 8).

Samples obtained from the budgerigars in Pretoria (B-PTA-1, B-PTA-2, B-PTA-3, and B-PTA-5) had similar digest profiles with fragments of approximately 410 bp, 225 bp and 50 bp in size (Fig. 2, lanes 2–4, 6), except for an additional fragment observed in sample B-PTA-4 with an approximate size of 370 bp (Fig. 2, lane 5).

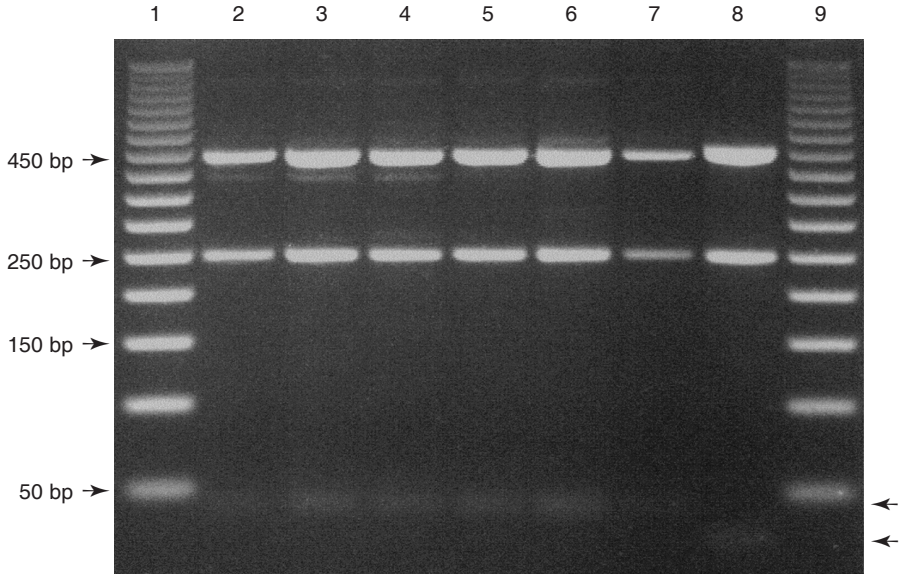

FIG. 1 Restriction digest profile for the samples obtained from ring-neck parakeets using the restriction enzyme *Hae*III. The digest products were loaded onto a 2.5 % agarose gel together with a 50 bp step ladder (Promega) [Lanes 1 and 9]. Samples P-BLM-1, P-BLM-2, P-BLM-3, P-BLM-4, P-BLM-5, P-BLM-6 and P-GEO were loaded in lanes 2–8 respectively. Arrows on the right hand side indicate the discrepancy found of the ~50 bp band between the six samples obtained from Bloemfontein (upper arrow) in comparison to the sample obtained from George (lower arrow)

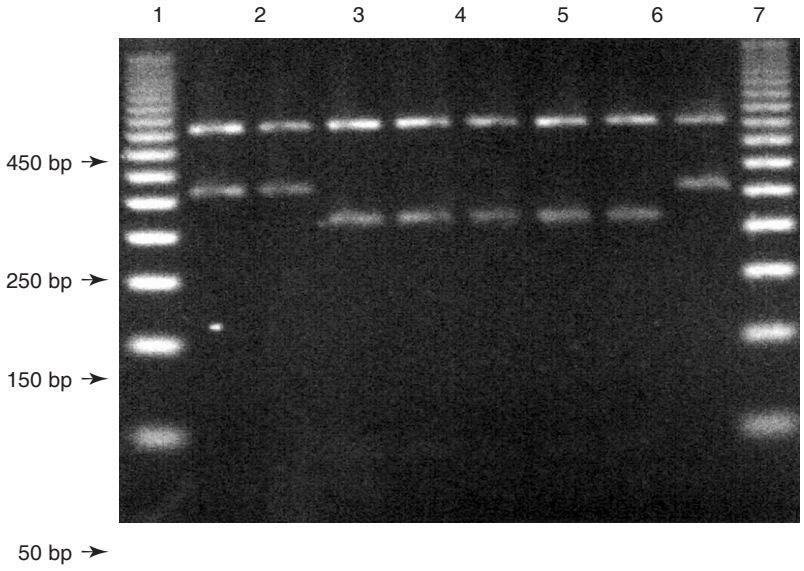

FIG. 2 Restriction digest profile for the samples obtained from budgerigars (Pretoria) using the restriction enzyme *Hae*III. The digest products were loaded onto a 2.5 % agarose gel together with a 50 bp step ladder (Promega) [Lanes 1 and 7]. The samples B-PTA-1, B-PTA-2, B-PTA-3, B-PTA-4 and B-PTA-5 (lanes 2–6 respectively) gave similar profiles with sample B-PTA-4 containing an additional fragment of approximately 370 bp in size. The fragments below the 50 bp marker were light in intensity and therefore more difficult to observe

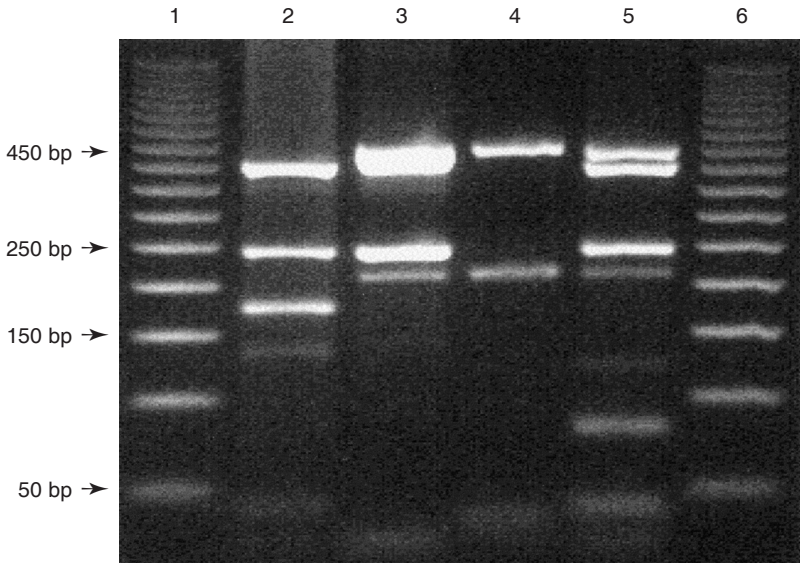

FIG. 3 Restriction digest profile for the samples obtained from budgerigars (Cradock) using the restriction enzyme *Hae*III showing multiple fragments. The digest products were loaded onto a 2.5 % agarose gel together with a 50 bp step ladder (Promega) [Lanes 1 and 6] as a molecular mass marker. The samples B-CRA-1, B-CRA-2, B-CRA-4 and B-CRA-5 were loaded in lanes 2–5 respectively

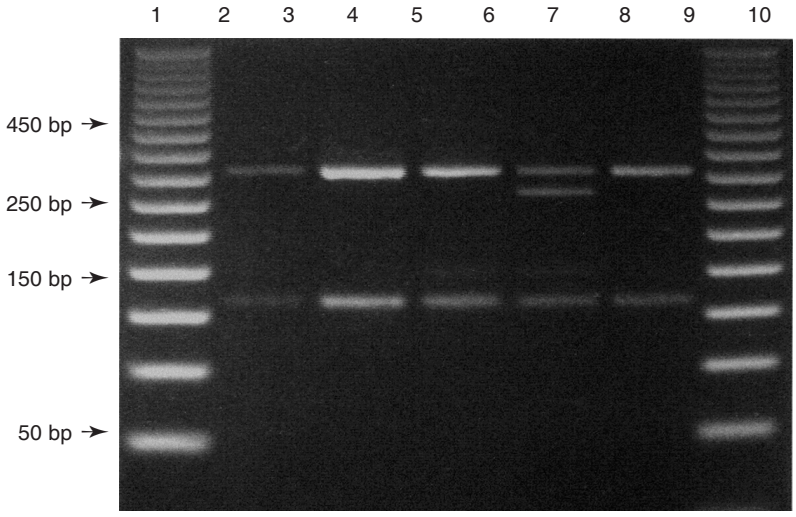

FIG. 4 Restriction digest profile for two randomly picked clones from each sample obtained from budgerigars (Cradock) using the restriction enzyme *Hae*III. The digest products were loaded onto a 2.5% agarose gel together with a 50 bp step ladder (Promega) [Lanes 1 and 10] as a molecular mass marker. Loading was performed as follows, B-CRA-1 [lanes 2 and 3], B-CRA-2 [lanes 4 and 5] B-CRA-4 [lanes 6 and 7] and B-CRA-5 [lanes 8 and 9]

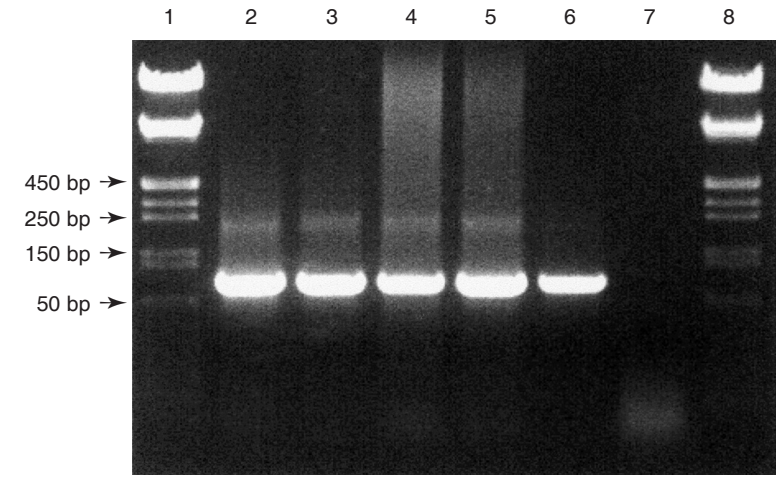

FIG. 5 PCR amplified products of PBFDV (P-BLM-4) from samples collected on filter paper, followed by DNA extraction after 24 [lane 2], 48 [lane 3], 72 [lane 4] and 96 h [lane 5]. Lane 6 contains the PCR product of a sample (P-GEO) that was received on commercially available specimen collection paper and processed after 1 month. Lanes 1 and 8, contains a molecular mass marker (λ *Eco*R1/*Hind*III), and lane 7 a negative control from a sample that was shown to be negative (B-CRA-3)

The digest profile obtained for the samples obtained from budgerigars in Cradock continuously resulted in an unusual profile (Fig. 3) that led to the possibility that more than one PCR amplicon (i.e. amplicons from genetically different viral isolates) of approximately 700 bp was present.

These amplicons were cloned into the vector pGEM™ TEasy, inserts were purified from two randomly selected clones for each sample, and digested with *Hae*III (Fig. 4). Clones from B-CRA-1 (Fig. 4, lanes 2 and 3) were similar in profile to each other but different from B-CRA-2 and B-CRA-4 [Fig. 4, lanes 4–7 respectively], which in turn shared a similar profile. Sample B-CRA-5 [Fig. 4, lanes 8 and 9] had a different profile for each clone; with one clone (Fig. 4, lane 8) sharing a similar profile with samples B-CRA-2 and B-CRA 4 and the second clone (Fig. 4, lane 9) sharing a profile with that of B-CRA-1 This profile indicates that at least two genetically different PBFDV isolates were present in these birds and, more importantly, that at least one bird contained both of these genetically different isolates.

The samples collected on filter paper all produced amplicons of the expected size (approximately 700 bp) and all amplicons, including the blood sample that was processed after one month of storage at –20 °C, showed the same intensity (Fig. 5). None of the products showed any non-specific amplification indicating that the filter paper collection methods is an easy and cost effective way to collect and process blood samples.

DISCUSSION

The PCR primer set PB F1 and PB R1 and primer set PB F2 and PB R2 amplified the expected products in all the samples tested except for one budgerigar that tested negative. The PCR amplicons obtained by amplification using primer set PB F2 and PB R2 were not used in further processes although this primer set also resulted in reproducible results. The reason for designing these primers and performing amplifications using them was to attempt to amplify the entire ORF1, which will be important for future studies and the potential development of a subunit vaccine.

The variation in the digest profiles between species and individuals suggests genetic variation in the sequences of the amplified region but it is still unknown if these variances indicate any differences in antigenicity or pathogenicity. Profiles for all the samples obtained from the different regions in the country were not similar to each other even though they were from the same species (budgerigars from Pretoria and Cradock). There may be a possibility that there is a relationship between regional distribution and genetic variation but this study does not give enough evidence to confirm or refute this.

Bassami *et al*. (2001) reported that there is a possibility that adaptation of particular genotypes to specific species may occur and that regional differences in strains may develop; they suggested that these differences would have a significant implication of the development of a universal PCR assay that would detect all strains of PBFDV. The genetic variation between these birds lends an important view in the production of a vaccine. If there are significant differences in the antigenicity or pathogenicity and other physiochemical characteristics of these isolates then it is important to develop a vaccine that can effectively protect a bird against any isolate or isolates that may infect it at any par-

ticular time. In the related porcine circovirus (PCV) two distinct genotypes have been identified (Todd 2000) and it is possible that similar genotypic differences have occurred in PBFDV.

The PCR tests performed were reproducible and confirm the occurrence of PBFDV infection in the ring-neck parakeets and budgerigars in the regions from where the samples were obtained. However, it was difficult to rule out the possibility of APV infection in the birds tested. Although a PCR test using primers to amplify the region coding for protein VPI of APV was performed and negative results were obtained, these results are not conclusive due to the lack of a positive control. A positive control was difficult to obtain as there were no reported cases of APV infection in South Africa at the time of study. This study then does not exclude the possibility of a concurrent infection of PBFDV and APV in the birds tested.

The method evaluated in this study for the collection of blood using filter paper was successful and did not have any negative effects on downstream processes. The results were reproducible and the PCR amplification was comparable to that performed using frozen blood samples. This method is simple, easy, does not require large amounts of blood (thus less stressful to the birds) and the samples do not require the use of anticoagulants, do not have to be frozen and are easy to transport. Most importantly however, it does not affect downstream processes. With all these advantages it is evident that the implementation of this method of sample collection is a suitable replacement to the more traditional methods of blood collection.

In conclusion, this study has shown the occurrence of PBFDV infection in ring-neck parakeets and budgerigars in South Africa by establishing and optimising a reliable PCR diagnostic test with reproducible results. The primers that were designed during this project were successful in producing the amplicon of expected size when used in the PCR test. Genetic variation between isolates from ring-neck parakeets and budgerigars is evident as well as genetic differences between isolates within individuals of the same species. However, the significance of these differences to PBFDV antigenicity, pathogenicity and other physiochemical characteristics must be investigated if an effective vaccine is to be produced. Blood sample collection on filter paper is easy to use, is less stressful to the birds and does not impact negatively on further processes.

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