Detection of beak and feather disease virus DNA in embryonated eggs of psittacine birds

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ABSTRACT: Psittacine beak and feather disease (PBFD) is a common viral disease of psittacine birds. The causative agent, beak and feather disease virus (BFDV) is a small circular single-stranded DNA virus belonging to the genus *Circovirus*. We report the detection of viral DNA predominantly in the heart, intestine and liver, but also in the testes and digestive organs. Additionally, BFDV transmission was investigated. We discuss the possibility that BFDV is transmitted horizontally. In our experiments, embryonated and non-embryonated budgerigar eggs were analysed for the presence of BFDV DNA. In 35.3% of non-embryonated and 20% of embryonated eggs viral DNA was detected, suggesting that BFDV can be transmitted horizontally and vertically.

Keywords: beak and feather disease virus (BFDV); vertical transmission; horizontal transmission; PCR; embryonated eggs; non-embryonated eggs; psittacine birds

Psittacine beak and feather disease virus (BFDV) is a member of the circovirus family which is divided into two genera, *Gyrovirus* and *Circovirus*. The latter one contains porcine circoviruses type 1 (PCV1) and type 2 (PCV2) as well as several avian circoviruses. In addition to BFDV (Pass and Perry, 1984; Todd, 2004), circoviruses infecting a wide range of non-psittacine birds such as pigeons (PiCV), geese (GoCV), ducks (DuCV), gulls (GuCV), finches (FiCV), starlings (StCV) and canaries (CaCV) have been identified (Phenix et al., 2001; Todd et al., 2001, 2007; Hattermann et al., 2003; Johne et al., 2006; Smyth et al., 2006). The main clinical symptoms of birds infected with a circovirus are described as symmetric feather loss and beak deformities (Todd, 2004). To date, there exists neither a cure for infected birds nor a preventative vaccination.

BFDV, a non-enveloped particle of 14–16 nm in diameter possessing isometric or spherical symmetry, contains a single-stranded DNA (ssDNA) genome of 1992–2018 nucleotides which contains seven open reading frames (Niagro et al., 1998). This virus is the causative agent of psittacine beak and feather disease (PBFD) which is one of the most commonly recognized viral diseases of wild and captive psittacine birds worldwide. In many psittacine species, the disease is characterized by a chronic, progressive, symmetrical feather dystrophy and occasional beak deformity (Pass and Perry, 1984). Typically, the first sign of PBFD is a replacement of normal down and contour feathers with dystrophic feathers that stop growing shortly after emerging from the follicle. If beak lesions develop, they may include palatine necrosis, progressive elongation and transverse or longitudinal fractures (Pass and Perry, 1984).

BFDV has been reported to infect more than 60 species of both wild and captive parrots (Rahaus and Wolff, 2003; Bert et al., 2005; Hsu et al., 2006). Virus prevalence is reported to be between 10 and 94% (McOrist et al., 1984; Raidal et al., 1993; Khalesi et al., 2005). Within the population of captive psittacine birds in Germany, a prevalence of 39.2% has been described (Rahaus and Wolff, 2003).

Thus far, the transmission of the virus has not been fully elucidated. Based on findings from other avian circoviruses, it is possible that BFDV can be

transmitted both horizontally and vertically. Since PiCV has been found in faeces, it is postulated to be transmitted horizontally through the ingestion of faecally contaminated material (Woods et al., 1993). Alternatively, horizontal transmission may be possible when adults feed their young. Recently, PiCV DNA has been detected in embryos before hatching also suggesting a vertical transmission (Duchatel et al., 2006).

The aim of the work presented here was to investigate the transmission of BFDV using PCR-techniques to detect viral DNA.

MATERIAL AND METHODS

PCR amplification of a BFDV genomic fragment

PCR analysis with the aim of detecting BFDV DNA was performed as described recently (Rahaus and Wolff, 2003) with some modifications. In brief, 500 ng of DNA was used as a template in a reaction mixture containing 50 pmol of each primer, 100µM dNTP, 3mM $MgCl_2$, 16mM $(NH_4)SO_4$, 67mM Tris/HCl (pH 8.8), 0.1% Tween-20, 3% DMSO and 2.5 IU Biotherm DNA-Polymerase (Genecraft, Germany). The primers were used as described by Ypelaar et al. (1999) and amplified a fragment of 717 bp in length in ORF1. Thermocycler conditions were set as follows: 4 min 94°C initial denaturation followed by 40 cycles of 30 s 94°C, 30 s 60°C and 90 s 72°C. The resulting products were separated on 1.6% agarose gels.

Sample preparation

DNA isolation from the feather calami was performed according to Rahaus and Wolff (2003).

After the dissection of dead birds and the preparation of organs, DNA was isolated from the respective tissue using the DNA blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

Both embryonated (10–12 days of incubation), and non-embryonated eggs were opened using a sterile scalpel; glair and yolk were separated and introduced into a phenol/chloroform extraction and ethanol was used for precipitation. Precipitated DNA was resolved in water and used for PCR. DNA derived from embryonic tissues was isolated using the DNA blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

RESULTS

In our first experimental approach we investigated, *post mortem* by PCR analysis, the presence of BFDV DNA in tissues from different organs of six parrots – four adult budgerigars (*Melopsittacus undulates*) and two young brown-headed parrots (*Poicephalus c. cryptoxanthus*). In all cases BFDV DNA was found to be present in the heart (6/6 cases). After cardiac tissue, BFDV DNA was detected most frequently in the liver (2/2 cases) and in the intestine (4/4 cases). It was detected less regularly in all other tissues tested. Interestingly, viral DNA was also found in the testes (2/3 cases) as well as in the upper respiratory/di-

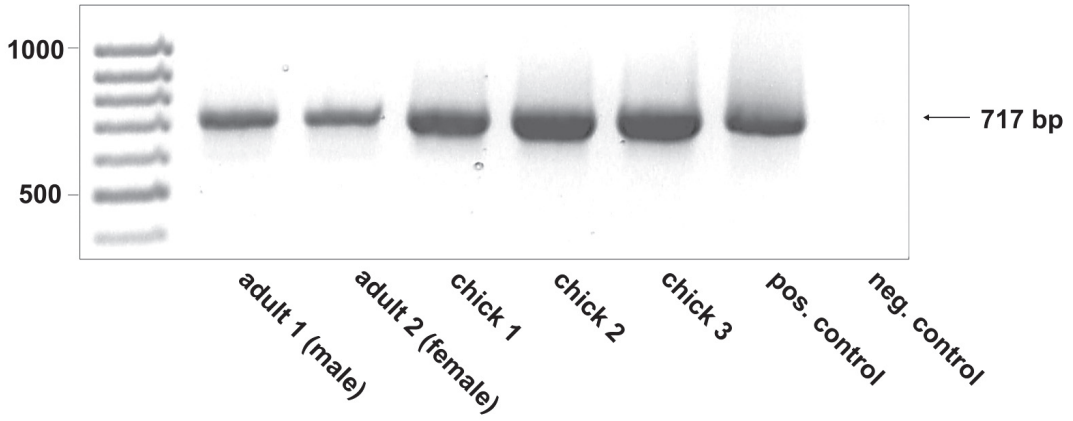

Figure 1. Detection of BFDV DNA isolated from feather samples of psittacine birds by PCR: analyses of a breeding pair of budgerigars and their three young. All birds were found to be BFDV positive

gestive tract (trachea 2/3 cases, crop 1/2 cases) and in the cloaca (2/4 cases). Although due to the age, size and condition of the carcasses not all organs could be prepared in all cases, our findings correlate significantly with previous reports (Pass and Perry, 1984; Smyth et al., 2001). They also adumbrate the possibility that BFDV can be transmitted through both vertical and horizontal routes.

Further investigations were performed by investigating whether nestlings (approximately four weeks of age) of BFDV positive parents were also positive for the virus. To this end, feather samples of different couples of budgerigars and their young were analysed. Template DNA for positive or negative controls was derived from birds (*Agapornis* sp.) with or without a clear manifestation of PBFD, respectively. Positive or negative BFDV states of the control material were confirmed by another independent laboratory. Figure 1 shows a representative example of results obtained from the analysis of one couple and their three young. All birds, parents and young, were positive for BFDV. Similar

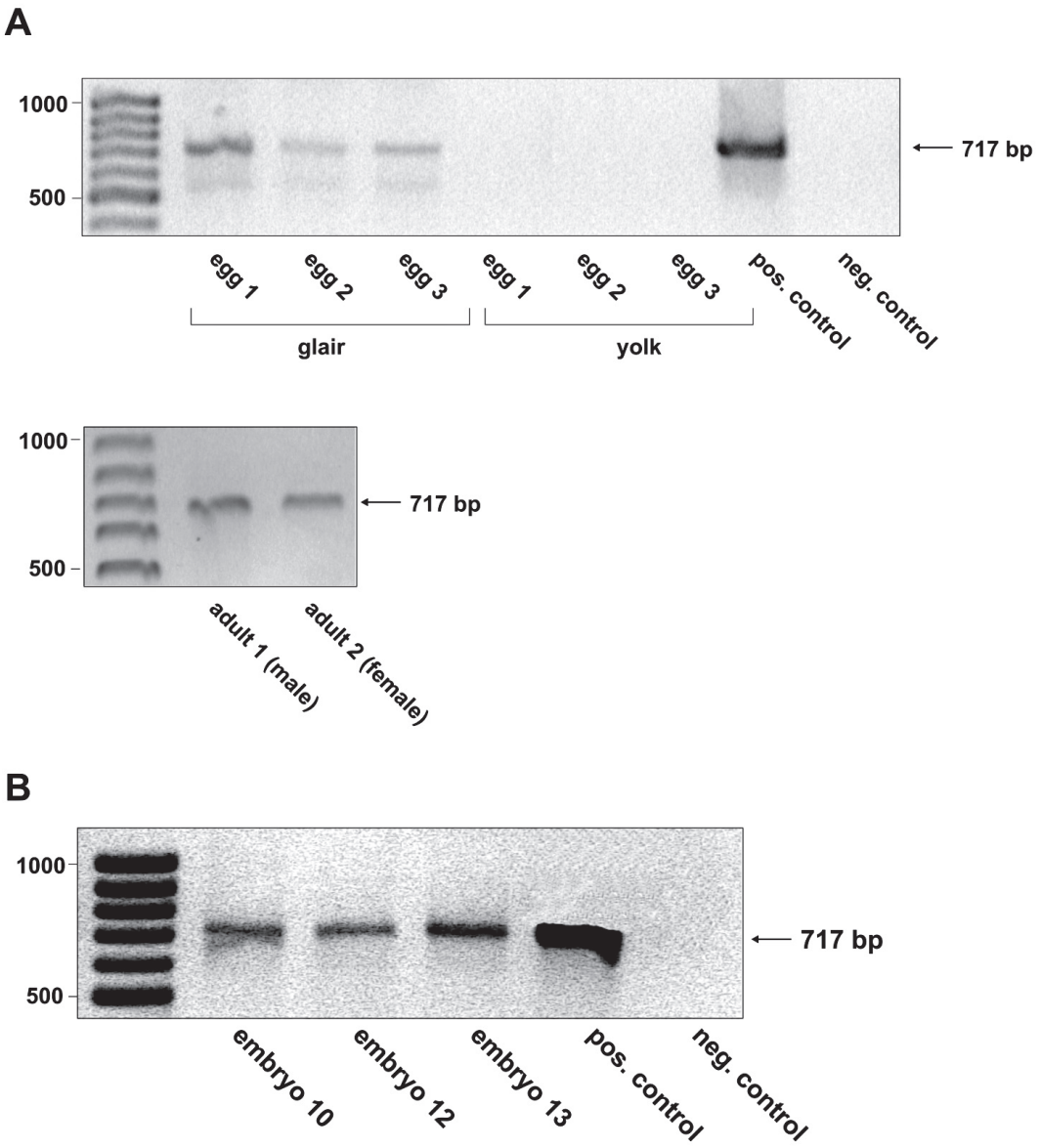

Figure 2. Detection of BFDV DNA in non-embryonated budgerigar eggs (A). After phenol extraction and PCR amplification, viral DNA was found in the glair of eggs but not in the yolk. The corresponding breeding pair was also confirmed to be positive for BFDV (A, lower panel). Detection of BFDV DNA in embryonated eggs (B). After 10–12 days of incubation, eggs were collected. Subsequent to DNA isolation, PCR was performed to detect viral DNA

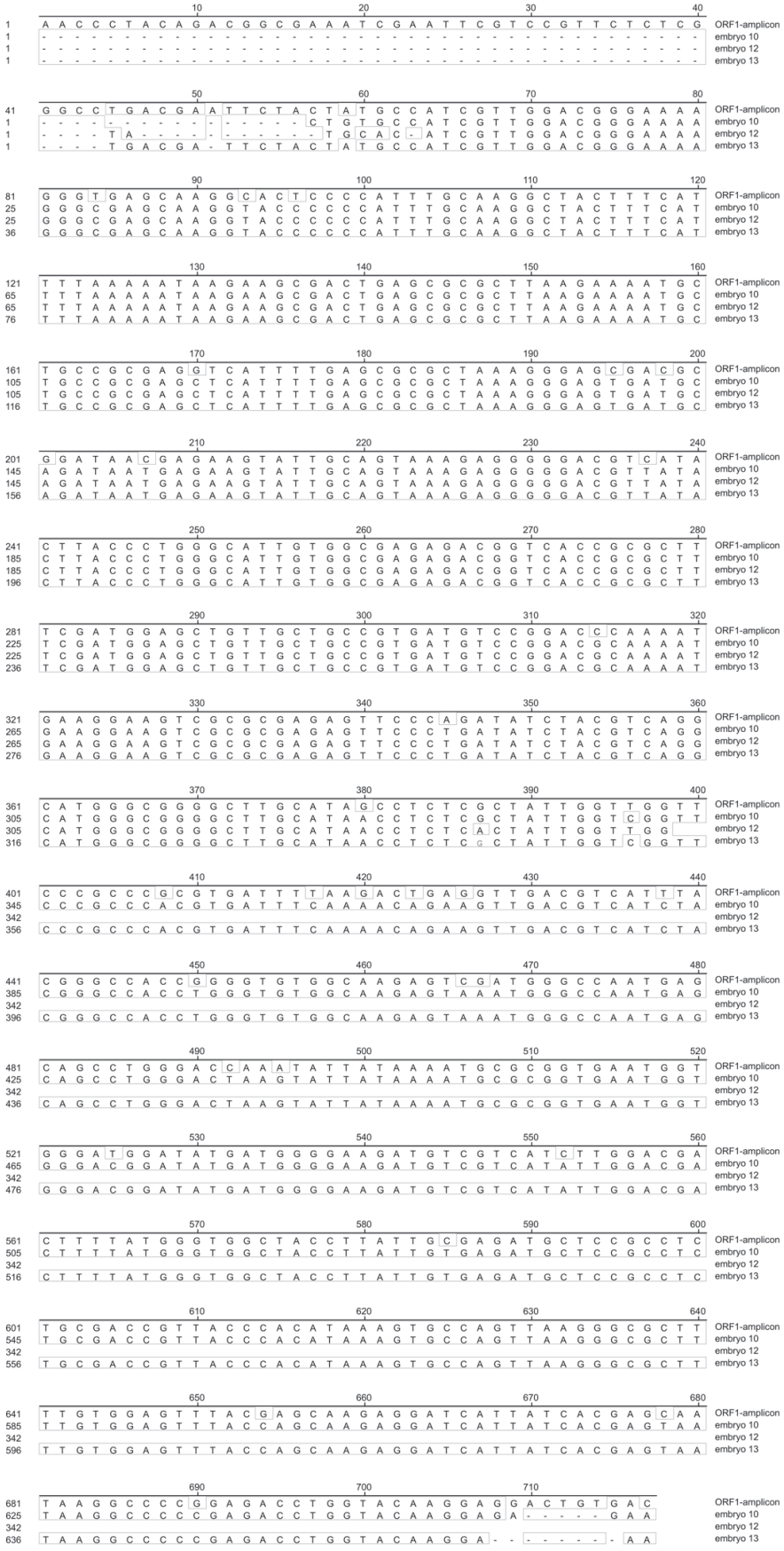

Figure 3. Alignment of BFDV sequences of the ORF1 control and of DNA isolated from embryos 10, 12 and 13

results were found after testing other adult birds and their respective young (data not shown). In summary, the data shown in Figure 1 indicate that BFDV has been transferred from the adult birds to the offspring.

To analyse the possibility of a vertical transmission of BFDV, we investigated the presence of viral DNA in embryonated and non-embryonated eggs. In the case of glair, 6 of 17 samples (35.3%) were found to be BFDV positive. A representative example is given in Figure 2A. It shows the PCR amplification of viral DNA isolated from the glair of three different eggs (all derived from the same clutch) as well as from feather samples of both parent birds, male and female, as controls. In the case of yolk, all samples were negative. Representative data derived from yolk analyses which are shown in Figure 2A were obtained from the same eggs which were the source of the positive glair.

In addition, embryonated eggs were also analysed. After 10 to 12 days of incubation, embryonated eggs were collected from breeding pairs. In 3 of 15 samples (20%) BFDV DNA was detected (Figure 2B). In order to confirm that the amplified DNA was indeed derived from the BFDV genome, PCR fragments were sequenced. Afterwards, sequencing data were aligned (Figure 3) with the sequence of the BFDV ORF1 fragment as given in GenBank (AF071878). On average, viral DNA isolated from embryos 10, 12 and 13 showed 95% sequence homology to the control ORF1 fragment and 98.4% homology with each other.

These findings suggest that embryos can also carry the virus lending credence to the hypothesis that vertical transmission of BFDV is possible.

DISCUSSION

The clinical symptoms of PBFD are now well characterized and much is known regarding the virus' pathology. T lymphocytes are the main target cells for avian circoviruses and particles have been detected in blood, macrophages, feathers, crop secretions, intestinal contents and/or faeces. Most avian circoviruses are pantropic and viral antigen in pigeon tissues has most commonly been detected in respiratory organs, including the trachea, pharynx and lung (Todd, 2004).

Surveys indicate a high prevalence of the virus in symptom-free populations of captive psittacine birds in Europe (Rahaus and Wolff, 2003; Bert et al., 2005). However, an understanding regarding the transmission dynamics of BFDV has remained elusive. Previously published data support the idea of horizontal transmission, predominantly via the inhalation of material derived from contaminated faeces or feather dust, since PiCV infections have been detected in cloacal swabs from young pigeons between 2 and 31 weeks of age (Todd et al., 2006). Additional findings suggest that young pigeons become infected during their stay in the rearing loft. It has been shown that cloacal swab samples from only 15.8% of birds were positive when they were 37 days old but all birds were positive when 51 days old (Duchatel et al., 2006).

Alternatively, Duchatel and co-workers (Duchatel et al., 2006) reported the detection of PiCV in embryonic pigeons but not in swabs taken from the crops of 64 adult birds which were feeding one- to 10-day-old squabs. Viral DNA appeared to persist in the ovary and testis of a small proportion of adult birds suggesting that it might be transmitted vertically (Duchatel et al., 2006). Data presented here correspond to these findings.

In summary, the findings presented strongly suggest that BFDV has the potential to be transmitted vertically. Since horizontal transmission has already been demonstrated to occur, it is likely that BFDV is transmitted via both routes, depending on the locus of persistence of the virus and the viral load during an acute viremia of the transmitting bird. These findings should impact on the design of monitoring systems as well as breeding programs.

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