Unusual fatal avian polyomavirus infection in nestling cockatiels (*Nymphicus hollandicus*) detected by nested polymerase chain reaction

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ABSTRACT: High mortality of nestling cockatiels (*Nymphicus hollandicus*) was observed in one breeding flock in Slovakia. The nestling mortality affected 50% of all breeding pairs. In general, all the nestlings in affected nests died. Death occurred suddenly in 4- to 6-day-old birds, most of which had full crops. No feather disorders were diagnosed in this flock. Two dead nestlings were tested by nested PCR for the presence of avian polyomavirus (APV) and *Chlamydophila psittaci* and by single-round PCR for the presence of beak and feather disease virus (BFDV). After the breeding season ended, a breeding pair of cockatiels together with their young one and a fledgling budgerigar (*Melopsittacus undulatus*) were examined. No clinical alterations were observed in these birds. Haemorrhages in the proventriculus and irregular foci of yellow liver discoloration were found during necropsy in the young cockatiel and the fledgling budgerigar. Microscopy revealed liver necroses and acute haemolysis in the young cockatiel and confluent liver necroses and heart and kidney haemorrhages in the budgerigar. Two dead cockatiel nestlings, the young cockatiel and the fledgling budgerigar were tested positive for APV, while the cockatiel adults were negative. The presence of BFDV or *Chlamydophila psittaci* DNA was detected in none of the birds. The specificity of PCR was confirmed by the sequencing of PCR products amplified from the samples from the young cockatiel and the fledgling budgerigar. The sequences showed 99.6–100% homology with the previously reported sequences. To our knowledge, this is the first report of APV infection which caused a fatal disease in parent-raised cockatiel nestlings and merely subclinical infection in budgerigar nestlings.

Keywords: budgerigar fledgling disease; *Melopsittacus undulatus*; parrots; viruses; PCR

Avian polyomavirus (APV) disease, formerly called budgerigar fledgling disease or French moult, is a common disease among nestling budgerigars (*Melopsittacus undulatus*). In non-budgerigar psittacine species, fatal infections occur in hand-raised nestlings. APV infection was first described in 1981 in budgerigars in the USA (Bozeman et al., 1981; Davis et al., 1981) and Canada (Bernier et al., 1981). Since then, it has been reported worldwide (Reece et al., 1992; Scott, 1994; Sandmeier et al., 1999; Ozmen and Dorrestein, 2004; Bert et al., 2005).

APV is an uncoated virus 40–48 nm in diameter (Bernier et al., 1981; Bozeman et al., 1981; Davis et al., 1981). Its genome consists of the circular double-stranded DNA (Lehn and Muller, 1986; Rott et al., 1988).

The course of the disease depends on the species and host's age and other, still largely unknown factors (Phalen et al., 2000). Most APV infections in fledgling and adult budgerigars result in subclinical forms and only a small percentage of infected birds develop a clinically apparent disease (Phalen et al., 1997, 2000). In enzootically infected flocks of budgerigars, the infection of nestlings breaks out within the first few days after hatching and prevalence can reach up to 100% (Phalen et al.,

1993, 1997). The highest mortality was observed in nestlings between 10 and 20 days of age (Bernier et al., 1981; Davis et al., 1981). Infected budgerigars that survive exhibit feather problems and shed the virus in their faeces and skin and feather dander for up to 6 months after infection. After that, the concentration of the virus in their tissues gradually decreases and virus shedding can completely diminish with the onset of sexual maturity and first breeding (Phalen et al., 1993, 1997, 2000).

APV infections in other non-budgerigar psittacine species are also common (Jacobson et al., 1984; Graham and Calnek, 1987). Macaws, conures, eclectus parrots (*Eclectus roratus*), ring-neck parakeets (*Psittacula krameri*), and lovebirds are the most frequently affected species (Phalen et al., 1997). The disease occurs especially in hand-raised nestlings between 3 to 14 weeks of age. Most infections in parent-raised nestlings and adults are asymptomatic (Phalen et al., 1997, 2000). In cockatiels (*Nymphicus hollandicus*), resistance to the clinically apparent disease is assumed (Phalen et al., 1997).

Several PCR protocols were developed for the detection of APV DNA in a host organism (Phalen et al., 1991; Johne and Muller, 1998) including the duplex shuttle PCR for the simultaneous diagnostics of APV and beak and feather disease virus (BFDV) (Ogawa et al., 2005). APV was detected in 0.79% of psittacine birds in Italy (Bert et al., 2005) and in none of the 85 asymptomatic non-budgerigar psittacines in Germany (Rahaus and Wolff, 2005). In Japan, an APV infection was found to be prevalent in 2.7% of asymptomatic psittacines (Ogawa et al., 2006). In Costa Rica, antibodies specific to APV were detected in 9.37% of the captive scarlet macaw (*Ara macao*).

In this study, an unusual fatal infection in parent-raised nestling and fledgling cockatiels is described.

MATERIAL AND METHODS

Birds

From spring to autumn 2004, high mortality of cockatiel nestlings occurred in one breeding flock of exotic birds in Central Slovakia. There were 14 breeding pairs of three to four years old cockatiels (*Nymphicus hollandicus*) kept in this flock. These birds were hatched and raised in this flock or bought as adults from other flocks. They bred healthy offspring in previous years. All birds were kept in outdoor facilities, where the contact with free-living birds was possible through the wire netting.

According to the information provided by the owner, deaths of nestlings occurred only in one pair of breeding cockatiels during the first nesting (7% morbidity), in two pairs during the second nesting (14%), and in seven pairs during the third nesting (50%). Adult cockatiels showed neither feather problems nor any other clinical symptoms of a disease. The nestling mortality reached 100% in affected nests. Most nestlings died within the first fifth days after hatching, had full crops, and did not manifest any clinically apparent disease symptoms. The administration of vitamins A, C, E, and Echinacea drops to the breeding birds during the third nesting cycle prolonged nestlings' survival. However, they did not stay alive for more than one week after having left the nests. Only a single fledgling originating from an affected nest in the third nesting cycle survived.

Other birds such as crimson rosellas (*Platycercus elegans*), lovebirds (*Agapornis* sp.), zebra finches (*Taeniopygia guttata*), red-rumped parrots (*Psephotus haematonotus*), and five pairs of budgerigars (*Melopsittacus undulatus*) were bred in the same aviary. All these species were without any symptoms of a disease and raised healthy offspring. Only fledglings of two breeding pairs of budgerigars, which settled in their nests for the fourth time during that season, were reluctant to fly. There was no problem even in zebra finches and red-rumped parrots that were kept together with the affected cockatiels in the same cages.

Examination and sampling

Samples from the two nestlings hatched in the second nesting cycle that died at five days of age were taken for the PCR testing. After the breeding ended, a four-month-old cockatiel hatched in the third nesting cycle, its parents, and a budgerigar fledgling reluctant to fly were submitted for examination. The young cockatiel was the only one that survived in an affected nest. Birds were clinically examined and cloacal swabs from the cockatiels were submitted for bacteriological cultivation. Further on, the cockatiel male, the female, their young one, and the fledgling budgerigar were anaesthetised

with a mixture of isoflurane and oxygen and exsanguinated. Blood samples from the cockatiels and the budgerigar were tested by the PCR for the presence of APV, BFDV and *Chlamydophila psittaci* DNA. All the birds were pathologically examined and tissue samples were taken for histopathological examination and PCR testing.

Histopathological examination

Samples of skin, liver, spleen, kidney, lungs, brain and testes or ovary were fixed in a 10% neutral water solution of formalin. The formalin fixed samples were processed routinely, embedded in paraffin, stained with haematoxylin and eosin, and examined for the presence of any histopathological changes (Department of Pathological Morphology, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic).

DNA isolation

Liver samples from two dead five-day-old cockatiel nestlings, blood samples from the male and female cockatiels, blood, liver, skin, spleen, kidney, testes, ovary, lungs, and brain samples from the young cockatiel, and liver, spleen and kidney samples of the budgerigar fledgling were taken for PCR testing.

The blood samples were allowed to clot and were processed immediately or stored at 5°C overnight. After the sedimentation of blood cells, DNA from 200 µl of plasma and 3 µl of buffy coat was extracted using an Invisorb Spin Blood Kit (Invitek, Berlin, Germany).

The tissue samples were stored frozen at –80°C until DNA extraction. DNA was extracted using an Invisorb Spin Tissue Mini Kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. The protocol was modified for a higher yield of extracted DNA. The modification consisted in two consecutive elution steps using 50 µl of elution buffer followed by centrifugation.

Nested PCR detection of APV

To ensure high sensitivity and specificity, a new nested PCR reaction was developed. In the first round of the PCR, forward primer Pol Sn 1646 (5'-CGT AGG TAT CAC GAG GTC G-3') and reverse primer Pol Asn 2328 (5'-ACC CAC GCT AAA GAA ATG G-3') were used to amplify a 683 bp long fragment in position bp 1646–2328 (numbering according to Stoll et al., 1993). In the second round of the PCR, forward primer Pol Sn 1911 (5'-GGA AAA GGA AGC TGC CCA CGA CC-3') and reverse primer Pol Asn 2286 (5'-CTC CCC CGT ATC CGT TAA ATG CG-3') were used for the amplification of a 376 bp long fragment in position bp 1911–2286. The reactions were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research, Watertown, Massachusetts). The first reaction was carried out in a total volume of 20 µl containing 7 µl of water, 1 µl of primers (12.5mM), 2 µl of template DNA, and 10 µl of PPP Master Mix with 100 U/ml Taq Purple DNA polymerase and 5mM $MgCl_2$ 400 µM of each dNTP (Top-Bio, Prague, Czech Republic). The amplification started with primary denaturation at 94°C for 4 min, continued with 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 25 s, and extension at 72°C for 45 s, and terminated with 2 cycles of annealing at 50°C for 30 s and final extension at 72°C for 1 min. The second reaction was carried out in a total volume of 21 µl containing 9 µl of water, 1 µl of primers (12.5 mM), 2 µl of the PCR product, and 10 µl of PPP Master Mix. The amplification started with primary denaturation at 94°C for 2 min, continued with 30 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 20 s, and extension at 72°C for 20 s, and terminated with 2 cycles of annealing at 63°C for 30 s and final extension at 72°C for 1 min. The products of both PCR reactions were separated by electrophoresis in 2% agarose gel at 8 V/cm for 20 min and were visualised with ethidium bromide. The product sizes were compared with the 2-Log DNA Ladder (New England Biolabs).

PCR detection of beak and feather disease virus (BFDV)

The PCR protocol described by Ypelaar et al. (1999) was used with some modifications. Forward primer PBFD 2 (5'-AAC CCT ACA GAC GGC GAG-3') and reverse primer PBFD 4+G (5'-GGT CAC AGT CCT CCT TGT ACC-3') were used to amplify a 718 bp long fragment in position bp 182–899 of ORF1. The reactions were carried out in a total volume of 20 µl containing 7 µl of water, 1 µl of primers (12.5mM), 2 µl of template DNA, and

10 µl of PPP Master Mix. The amplification started with primary denaturation at 94°C for 4 min, continued with 41 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, and terminated with 4 cycles of annealing at 55°C for 30 s and final extension at 72°C for 1 minute.

Nested PCR detection of *Chlamydophila psittaci*

The nested PCR described by Messmer et al. (2000) was used with some modifications. In the first round of the PCR, forward primer Chlr Sn 59 (5'-ACG GAA TAA TGA CTT CGG-3') and reverse primer Chlr Asn 495 (5'-TAC CTG GTA CGC TCA ATT-3') were used for the amplification of a 437 bp long fragment. In the second round of the PCR, forward primer ChlP Sn 64 (5'-ATA ATG ACT TCG GTT ATT ATT-3') and reverse primer ChlP Asn 191 (5'-TGT TTT AGA TGC CTA AAC AT-3') were used to amplify a 128 bp long fragment. The first reaction was carried out in a total volume of 20 µl containing 7 µl of water, 1 µl of primers (12.5mM), 2 µl of template DNA, and 10 µl of PPP Master Mix. The amplification started with primary denaturation at 94°C for 4 min, continued with 35 cycles of denaturation at 94°C for 30 s, annealing at 44°C for 20 s, and extension at 72°C for 45 s, and terminated with 4 cycles of annealing at 42°C for 30 s and final extension at 72°C for 1 min. The second reaction was carried out in a total volume of 17 µl containing 7 µl of water, 1 µl of primers (12.5mM), 1 µl of the first PCR product, and 8 µl of PPP Master Mix. The amplification started with primary denaturation at 94°C for 2 min, continued with 7 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 25 s, and extension at 70°C for 25 s, and 36 cycles of denaturation at 80°C for 30 s, annealing at 42°C for 25 s, and extension at 70°C for 25 s, and terminated with 4 cycles of annealing at 40°C for 30 s and final extension at 70°C for 1 minute.

Sequence analysis

The APV fragment, amplified from the young cockatiel's blood sample during the first round of the PCR, was sequenced in both directions. The APV fragment, amplified from the budgerigar fledgling's liver sample during the first round of the PCR, was sequenced in one direction only (Genomac International, Prague, Czech Republic). Sequencing was done with the help of the DYEnamic ET Dye Terminator Kit (Amersham Biosciences) and was analysed with the MegaBACE 1000 Genetic Analyser (Amersham Biosciences).

RESULTS

The clinical examination revealed neither feather problems nor any other clinical symptoms of a disease, except for a slightly impaired body condition of the fledgling budgerigar. *Escherichia coli* was cultivated from the cloacal swabs of the female and young cockatiel and *Streptococcus alfa-haemolyticus* was identified in the swab cultivations of the male.

The pathological examination of two cockatiel nestlings which had died during the second nesting cycle revealed only advanced tissue autolysis. Their crops were full of food. In the young cockatiel, hatched in the third nesting cycle, irregular foci of yellow discoloration of liver and proventriculus haemorrhages were discovered. There were no gross lesions in the adult cockatiels, except for the yellowish discoloration of liver in the female. The fledgling budgerigar was in a slightly impaired body condition. Fragile liver consistence, yellowish liver margins, and haemorrhages in the proventricular wall were also found. There were no feather problems observed in any of these birds.

Liver necroses and signs of acute haemolysis were revealed by microscopy in the young cockatiel. No microscopic changes were present in the female cockatiel, except for small necroses in the liver. In the male, marked proliferation of lymphocytes of spleen, liver and lungs, marked gliosis, and neuronal degeneration of the brain were observed. The histopathological examination of the fledgling budgerigar revealed confluent necroses of the liver and haemorrhages under the epicardium, in the myocardium, and in the kidney interstitium.

In the liver samples from two dead cockatiel nestlings hatched in the second nesting cycle, the nested PCR for APV detection amplified a 683 bp long fragment during the first round and a 376 bp long fragment during the second round. PCR detection of BFDV and nested PCR detection of *Chlamydophila psittaci* did not amplify any specific fragments in any of the liver samples.

The nested PCR results of the APV detection in the blood samples from the cockatiels are pre-

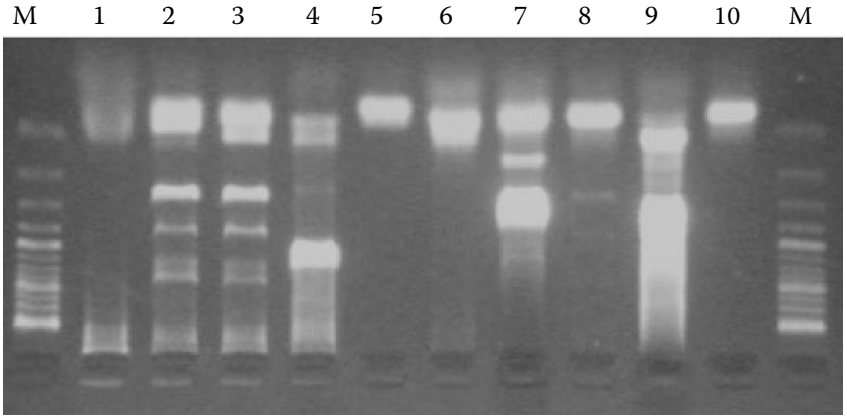

Figure 1. The results of the nested PCR for the detection of APV in blood samples from the young cockatiel hatched in the third nesting cycle and its parents in 2% agarose gel. Lanes 1–5 = the first round of nested PCR; lanes 6–10 = the second round of nested PCR. Lanes 1 and 6 = male; lanes 2 and 7 = young; lanes 3 and 8 = female; lanes 4 and 9 = positive control; lanes 5 and 10 = negative control. Lanes M = weight marker

sented in Figure 1. The fragment of approximately 683 bp was not amplified in any of the blood samples during the first round of nested PCR. The second round amplified a 376 bp long fragment in the blood sample from the young cockatiel. The adult cockatiels were negative for the presence of the APV DNA in blood. All three cockatiels were tested negative for BFDV and *C. psittaci* (Figures 2 and 3). Subsequently, the tissue samples of the young cockatiel and the fledgling budgerigar (both positive for APV in blood samples) were tested for the presence of APV. The cockatiel's spleen, heart, and brain samples were strongly positive, and the skin, liver, ovary, and kidney samples were moderately positive (Figure 4). Even though the lung sample was negative in the first round of nested PCR, the second round

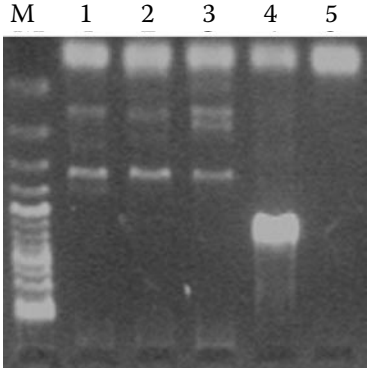

Figure 2. The results of the PCR for the detection of BFDV in blood samples from the young cockatiel hatched in the third nesting cycle and its parents. Lane 1 = male; lane 2 = young; lane 3 = female; lane 4 = positive control; lane 5 = negative control. Lane M = weight marker

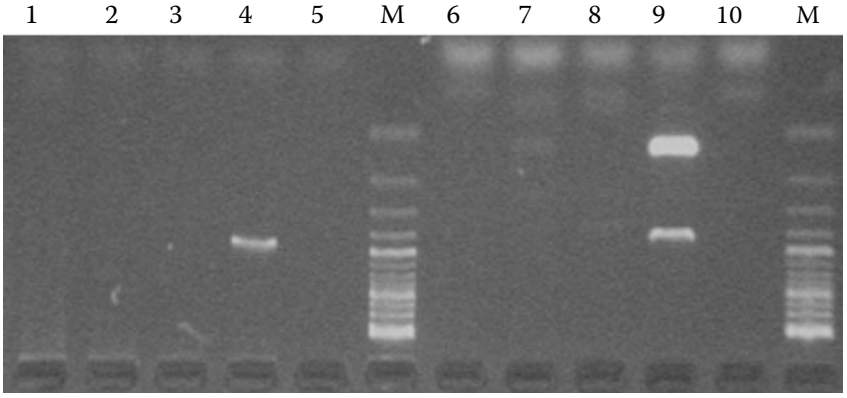

Figure 3. The results of the nested PCR for the detection of *Chlamydophila psittaci* in blood samples from the young cockatiel hatched in the third nesting cycle and its parents. Lanes 1–5 = the first round of nested PCR; lanes 6–10 = the second round of nested PCR. Lanes 1 and 6 = male; lanes 2 and 7 = young; lanes 3 and 8 = female; lanes 4 and 9 = positive control; lanes 5 and 10 = negative control. Lanes M = weight marker

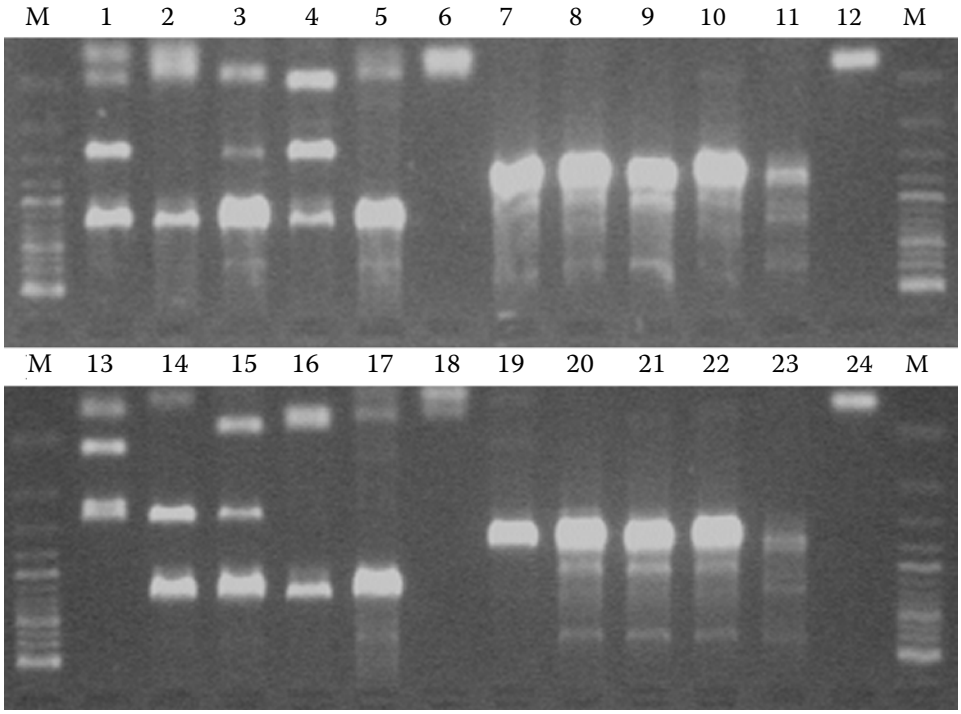

Figure 4. The results of the nested PCR for the detection of APV in tissue samples from the young cockatiel hatched in the third nesting cycle. Lanes 1–6 and 13–18 = the first round of nested PCR; lanes 7–12 and 19–24 = the second round of nested PCR. Lanes 1 and 7 = skin; lanes 2 and 8 = liver; lanes 3 and 9 = spleen; lanes 4 and 10 = kidney; lanes 13 and 19 = lungs; lanes 14 and 20 = heart; lanes 15 and 21 = brain; lanes 16 and 22 = ovary. Lanes 5, 11, 17 and 23 = positive control; lanes 6, 12, 18 and 24 = negative control. Lanes M = weight marker

produced strong positive signals in all the samples including the lung sample. The results of tissue samples from the fledgling budgerigar are summarized in Figure 5. In the first round of nested PCR, the liver sample was strongly positive, whereas the spleen and kidney samples were moderately positive and negative, respectively. All these tissue samples were strongly positive in the second round.

Products of the first round of nested PCR for APV detection in the young cockatiel and fledgling

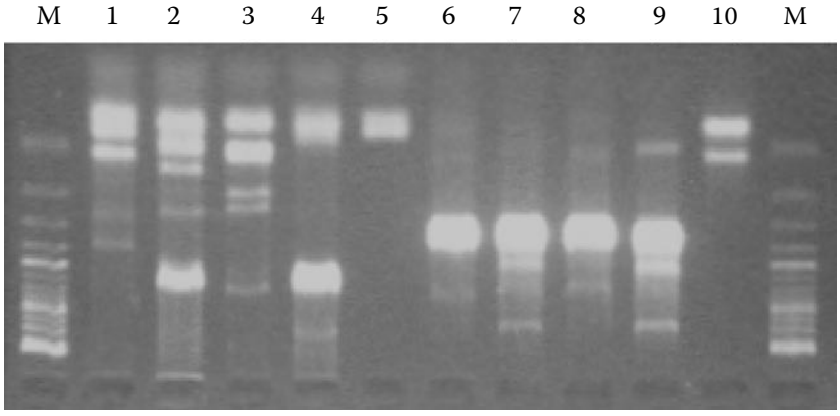

Figure 5. The results of the nested PCR for the detection of APV in tissue samples from the fledgling budgerigar. Lanes 1–5 = the first round of nested PCR; lanes 6–10 = the second round of nested PCR. Lanes 1 and 6 = kidney; lanes 2 and 7 = liver; lanes 3 and 8 = spleen; lanes 4 and 9 = positive control; lanes 5 and 10 = negative control. Lane M = weight marker

budgerigar were sequenced and the sequences were compared with those published previously using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The comparison of both sequences (NH-MS 1 from the young cockatiel and MU-MS 1 from the fledgling budgerigar) showed 100% homology with sequences M20775 (Rott et al., 1988), AF118150 (Lafferty et al., 1999) and AF241168 (Rott et al., 1988) and 99.6% (2 changes) homology with sequences AF241170 and AF241169 (Rott et al., 1988).

DISCUSSION

In non-budgerigar psittacine species, the APV induced disease is typically characterized by deaths in hand-raised nestlings. Macaws, conures, eclectus parrots, ring-neck parakeets, and lovebirds are the most frequently affected species (Phalen et al., 1997). The clinically apparent disease is rarely reported in APV infected cockatiels and it affects hand-raised nestlings only (Jacobson et al., 1984; Graham and Calnek, 1987). Moreover, high prevalence of APV infection and virus shedding lasting for more than 18 months were described in asymptomatic cockatiels. Based on these findings, possible tolerance of cockatiels to the APV caused clinical disease was discussed (Phalen et al., 1997). In contrast to these findings, high mortality occurred in the parent-raised nestling and fledgling cockatiels in this study. The number of affected nests increased during the breeding season from one (7%) during the first nesting through two (14%) during the second nesting to 7 out of 14 (50%) during the third nesting cycle. The nestling mortality reached 100% in almost all affected nests. The nestlings died at a very early age, most of them between the fourth and sixth day of their life. The nested PCR detected the presence of APV. In the parent-raised non-budgerigar psittacine nestlings, deaths could possibly occur when they are simultaneously infected with APV and BFDV or APV and *Chlamydophila psittaci*. Nevertheless, such simultaneous infection was excluded by the negative nested PCR results for both, BFDV and *Chlamydophila psittaci*.

In the budgerigars, no clinically apparent problems occurred during the breeding season, except for the reluctance of some of the fledglings to fly. This was noticed in two pairs during the fourth nesting cycle. The nested PCR testing of one of the fledglings revealed the presence of APV, which could be a possible cause of its weakening. It is not, however, clear why the mortality did not occur in these budgerigars as a result of APV infection, whereas mortality was high in the cockatiels.

Gross lesions in the young infected birds hatched in the last breeding cycle were yellow discoloration of the liver and haemorrhages in the proventricular wall. Such lesions are commonly seen in birds with APV infection (Bernier et al., 1981; Bozeman et al., 1981; Davis et al., 1981; Graham and Calnek, 1987). No feather disorders were observed in any of the examined birds, including the dead cockatiel nestlings hatched in the second nesting cycle.

The histological examination of the young birds at the end of the breeding season revealed necroses in the liver and acute haemolysis in the young cockatiel and confluent necroses and haemorrhages in many organs of the fledgling budgerigar. These changes are consistent with the changes described in APV infected birds previously (Bernier et al., 1981; Bozeman et al., 1981; Davis et al., 1981; Graham and Calnek, 1987). Changes seen in the male such as proliferation of lymphocytes, gliosis, and neuronal degeneration are more typical of the paramyxovirus infection.

The negative results of the nested PCR for APV in the adult cockatiels indicate that either they are resistant to the APV infection or the course of the infection is transient and the examined cockatiels had a chance to eliminate the virus. This is in contrast with the observations of Phalen et al. (1997), who described long-lasting persistence and shedding of APV in cockatiels.

The presence of APV was proved by the nested PCR in the young cockatiel raised by the adult pair in the third nesting cycle. The PCR product amplified from its blood sample was detected in the second reaction only. This was likely due to the low amount of the virus in blood, which could only be detected by highly sensitive nested PCR. The virus amount was probably higher in the tissues of the young birds where the APV DNA could already be detected in the first round of PCR. The lungs of the young cockatiel were the only exception.

Both sequences amplified by the nested PCR matched those described before (Rott et al., 1988; Lafferty et al., 1999) and we did not discover any previously unreported substitution. Our sequences covered, however, only two fifths of the VP1 ORF and only one seventh of the VP2 ORF, thus, there could be differences in the other parts of the APV

genome. It was assumed that a specific change in the ORF coding VP2/VP3 could modify the host specificity or pathogenicity in different host species. For instance, a substitution of glycine for alanine or valine at amino acid position 221 of VP2 which blocked the viral replication in chicken embryo fibroblasts (CEF) but not in Muscovy duck embryo fibroblasts (MDEF) was reported (Stoll et al., 1994). The authors speculated that a mutation in this position might interfere with the uncoating or nuclear localization of viral DNA in CEF cultures. The host restriction due to a mutation in this or other positions of the VP2/VP3 or VP1 ORF could explain why the APV infection was fatal in the cockatiels but only subclinical in the budgerigars.

Interestingly, this is not the first report of fatal APV infection in cockatiels in Slovakia. Literak et al. (2006) described a similar unusual case in one aviary in Eastern Slovakia during the winter and spring 2003/2004. The infection was fatal in nestling cockatiels up to 7 days of age with deaths occurring without any preceding clinical symptoms. In contrast to our case, a typical form of the disease with deaths between 14 and 21 days of age and feather disorders of surviving individuals was observed in budgerigars. However, the presence of other possible infectious agents like BFDV or *Chlamydophila psittaci* was not investigated in that case, even though they could influence the course of the disease. According to other epizootological studies, an infection with BFDV or *C. psittaci* is more probable than an APV infection. In Italy, APV was found in 0.79% of psittacines, compared to BFDV, which was detected in 8.05% of psittacine birds (Bert et al., 2005). In Germany, no APV positive sample was detected in asymptomatic psittacines in one study (Rahaus and Wolff, 2005), but 39.2% prevalence of BFDV was found in another study (Rahaus and Wolff, 2003). *C. psittaci* was found in 6.2% of psittacine birds in a long-term study in Slovenia, but with decreasing tendency resulting in 1.3% prevalence in 2001 (Dovc et al., 2005). However, neither BFDV nor *C. psittaci* was detected in our study. It is therefore suggested that the mortality in cockatiels was caused by the APV infection only.

The occurrence of the unusual fatal APV infection in cockatiels, which resembled the APV infection usually described in budgerigars, and only subclinical infection with the same virus in budgerigars indicate the necessity of more intensive research of mutations in the VP1 and VP2/VP3 ORFs and their possible role in host specificity or pathogenicity.

Acknowledgements

We would like to thank Prof. MVDr. Roman Halouzka, DrSc., dipl. EBVP for the evaluation of histopathological findings.

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Received: 2006–03–23
Accepted after corrections: 2007–04–17

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