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Original Scientific Article

DETECTION AND MOLECULAR CHARACTERIZATION OF A PIGEON VARIANT OF AVIAN PARAMYXOVIRUS TYPE 1 VIRUS (PPMV-1) FROM A BLACKBIRD *(TURDUS MERULA)*

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Summary: Cloacal and tracheal swabs taken from a dead blackbird *(Turdus merula)* were investigated as part of increased virological monitoring of wild birds during 2006, for the purpose of early detection of highly pathogenic avian influenza H5N1 virus. A pigeon variant of avian paramyxovirus type 1 virus (PPMV-1) was isolated by inoculation of the cloacal swab into the allantoic cavity of 10-day-old embryonated specific-pathogen-free chicken eggs. Molecular characterization of the isolate was performed by reverse transcription PCR and sequencing of the partial fusion (F) protein gene, including the region encoding the cleavage activation site of F protein. The PPMV-1 virus isolated from the blackbird shared the highest partial F gene nucleotide and amino acid sequence identity with PPMV-1 viruses isolated from the free-living and domestic pigeons in Slovenia between 2004 and 2006. The PPMV-1 strain isolated from the blackbird was classified, together with other PPMV-1 viruses associated with the ongoing panzootic in pigeons, into linage 4 sublineage 4b, group 4bii. The PPMV-1 virus isolated from the blackbird has a ^{112}RRQKRF^{117} sequence, associated with virulent avian paramyxovirus type 1 viruses (Newcastle disease viruses), at the F protein cleavage site. To the best of our knowledge this is the first description of the detection of a PPMV-1 virus in a blackbird.

Key words: blackbird (*Turdus merula*); avian paramyxovirus type 1; NDV; F gene sequences; phylogenetic analysis

Introduction

Avian paramyxovirus type 1 virus (APMV-1) of pigeons (PPMV-1) is an antigenic variant of Newcastle disease virus (NDV) of chickens, which causes an autonomous Newcastle disease (ND)-like infectious disease of pigeons (1). The APMV-1 viruses, including PPMV-1, are members of the genus *Avulavirus* (2) within the family *Paramyxoviridae* (3).

ND, included in the Office International des Epizooties (OIE) listed diseases, is a highly contagious and devastating avian disease and, in spite of control measures and vaccination, often manifests itself in epizootics (4). Clinical signs may vary from extremely mild respiratory or enteric diseases (avirulent viruses) to severe systemic infection resulting in high mortality (virulent viruses) and characterized by very rapid spread (5).

The amino acid motif at the precursor fusion protein (F_0) cleavage site has the major influence on the pathogenicity of APMV-1 viruses (6, 7). Different pathotypes of APMV-1 viruses are characterized by their amino acid sequence around the cleavage-site of F_0 (8), which require post-translational cleavage by the host proteases to produce disulphide-linked active F_1 and F_2 subunits of the F protein (9).

Molecular characterizations of APMV-1 strains have mainly considered the F and M genes. The most studied molecular pattern has been the F protein cleavage site (10, 11, 12).

As one of the major antigenic determinants of NDV, the F protein is likely to display greater genetic variation than internal genes. This characteristic is important for studying fairly closely related populations, in which a more conserved gene may show in-

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sufficient sequence variation to allow evolutionary theories to be inferred (13).

Nine NDV genotypes have been described for epizootic NDV strains worldwide (14, 15, 16). Most PPMV-1 strains have been classified into subgroup VIb within genotype VI of NDV by phylogenetic analysis of the region, including the F protein cleavage site (1, 17).

Nine NDV genotypes have recently been reclassified into six broadly distinct lineages (1 to 6) (5). PPMV-1 viruses associated with the ongoing panzootic in pigeons have been placed into sublineage 4b of lineage 4 (13).

Groups or lineages created by phylogenetic analysis appear to be largely congruent and reflect the degree of genetic diversity of NDV and epidemiological associations, such as geographical and/or temporal restrictions of the viruses, the origin of the viruses and the spread of the outbreaks (1).

PPMV-1 strains caused outbreaks among racing and show pigeons in Europe in 1981 and re-emerged in 1985, causing a panzootic that continues to this day (1, 13, 18, 19, 20, 21, 22,). The origins of the panzootic in pigeons appeared to be in the Middle East during the late 1970s and the evidence for this was the fortuitous isolation of PPMV-1 virus in Iraq in 1978 (23). Clinical signs of the infection of pigeons generally include a series of nervous disorders: paralysis, torticollis and watery green diarrhea (24).

Most PPMV-1 strains have had reduced virulence for chickens (25, 26, 27, 28) but, in the most cases, PPMV-1 isolates have increased their virulence for chickens after passages (25, 29, 30).

In addition to pigeons, doves and chickens, PPMV-1 viruses have also been isolated from kestrels, falcons, cockatoos, budgerigars, pheasants, swans and grey partridges (19, 20, 31, 27, 13, 32). Isolation of PPMV-1 virus from a passerine bird (Passeriformes), a robin (*Erithacus rubecula*), has recently been reported (33). Passerine birds are not generally considered important in the epizootiology of APMV-1 viruses (34). Nevertheless, recently published data have shown that passerine birds (an order with over 5000 individual species) can sporadically be infected with APMV-1 viruses (35).

Materials and methods

Isolation of the virus

Cloacal and tracheal swabs taken from a dead blackbird (*Turdus merula*) were submitted to the Institute of Poultry Health, Veterinary Faculty, University of Ljubljana as a part of increased virological monitoring of wild birds during 2006, for the purpose of early detection of the highly pathogenic avian influenza (HPAI) H5N1 virus.

Isolation of the virus was performed by the inoculation of cloacal and tracheal swabs, obtained from the dead blackbird, into the allantoic cavity of 9-to-10-day old embryonated specific-pathogen-free chicken eggs (Lohman, Cuxhaven, Germany) as described by Alexander (36). Briefly, 2ml of sterile phosphate-buffered saline, including antibiotics, 5,000IU/ml of penicillin, 5,000µg/ml of streptomycin and antimycotics, 12.5µg/ml of amphotericin B (Gibco, Paisey, UK) was added to the dry cloacal and tracheal swab. Swabs were vortexed and centrifuged at 1000 x *g* for 10 min. Next, 0.2 ml of supernatant was inoculated into the allantoic cavity of 9-to-10 day-old embryonating chicken eggs. The inoculated eggs were maintained at 37°C and candled daily. We chilled eggs with dead embryos (as they arose) and all eggs were chilled 5 days post inoculation. Allantoic fluids were collected and tested for hemagglutination activity. Isolates were identified through a haemagglutination inhibition test by using APMV-specific antiserums (APMV-1 to APMV-9, excluding APMV-5) and AIV-specific antiserums (H1N to H16N) (36) provided by Veterinary Laboratory Agency, Weybridge, UK. Infective allantoic fluids were stored at -70°C.

RNA extraction, reverse transcription, PCR, and nucleotide sequencing

RNA was extracted from infectious allantoic fluid by QIAamp Viral RNA Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

A region of approximately 454-bp of the F gene of APMV-1, including the F protein cleavage site, was amplified by the oligonucleotide primer pair: F-OPU: 5'-TTG A**Y**G GCA G**R**C CTC TTG C-3' and F-OPL: 5'-TGC ATC TTC CCA ACT GCC ACT-3' (37), with two modifications of the primer F-OPU indicated in bold.

OneStep reverse transcriptase (RT)-PCR Kit (Qiagen, USA) was used for genomic RNA amplification. RT-PCR was performed by uninterrupted thermal cycling with the following program: 30 min at 50°C for RT; RT inactivation at 95°C for 15 min was followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min.

The amplified products were analyzed by electrophoresis on a 1.8% agarose gel stained with the ethidium bromide. The DNA fragments were purified with a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). Direct double-stranded nucleotide sequencing was completed by an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Woolston, UK) and oligonucleotide primers used for RT-PCR. Reactions were analyzed with an ABI3730xl Genetic Analyzer (Applied Biosystems).

Sequence analysis

The genetic relationship between the partial nucleotide sequences of the F gene of APMV-1 isolated from the blackbird, SLO/12/06 and respective gene sequences of APMV-1 isolates published in GenBank was evaluated.

MEGA 3.1 software (38) was used for editing the nucleotide sequences and deducing the amino acid sequences. Nucleotide and deduced amino acid sequences were aligned with ClustalW software (39). Phylogenetic analyses were constructed with MEGA 3.1 software using the neighbor-joining method with the Kimura-2 parameter substitution model and 1000 bootstrap replicates to assign a confidence level to branches.

Sequences used for pairwise comparisons on F gene

PITTDO0177 (AY175759); PDECT95200 (AY471772); PDECT95204 (AY175752); PTRBU95211 (AY471773); PUKBR84261 (AY471853); PITDO00289 (AY471846); PITPH95294 (AY471839); PITDO01321 (AY471742); PITTD96334 (AY471843); PAEKE99364 (AY471785); PAEKE98373 (AY471783); PILSW01382 (AY471787); PAEKE98398 (AY471784); PUKDO96438 (AY471817); PUKPH96441 (AY471818); ACAGL90270 (AY135756); Q-GB506/97 (AF109887); FIVi1001/96/1 (AF091623); Q-GB445/97 (AF109886); HFRDK77188 (AY135758); PUKPI88224 (AY471830); PUKPI93396 (AY471816); PUKPI89229 (AY471826); PUKPI91233 (AY471800); PUKPI90236 (AY471798); PITTD96334 (AY471843); PITPI96407 (AY471841); PITPH95294 (AY471839); SLO 349/01 (DQ007545); SLO 912/08 (GU002444); SLO 17/04 (GU002428); SLO 75/06 (GU002442); SLO 218/05 (GU002434); SLO 802/05 (GU002438); SLO 718/05 (GU002436); SLO 872/04 (GU002432) and SLO 263/04 (GU002430).

The accession number of the strain SLO/12/06 is GU002440.

Results

Virus isolation

A haemagglutinating agent was isolated by inoculation of the cloacal swab taken from a dead blackbird (*Turdus merula*) into the allantoic cavity of 10-day-old embryonated SPF chicken eggs. The isolate was identified as the APMV-1 virus through a haemagglutination inhibition test by using APMV specific antiserums (36), provided by the Veterinary Laboratory Agency, Weybridge, UK. The presence of avian influenza viruses (AIV) was excluded through a haemagglutination inhibition test.

Molecular characterization of isolate

RNA was extracted from infectious allantoic fluid and a region of approximately 454-bp was amplified by the RT-PCR specific for the F gene region of APMV-1 viruses.

Comparison of the obtained nucleotide and deuced amino acid sequences of the APMV-1 virus isolated from a blackbird in 2006, SLO/12/06, showed that the isolate shared the highest partial F gene nucleotide and amino acid identities with the PPMV-1 strains isolated from the free-living and domestic pigeons in Slovenia between 2004 and 2006. The nucleotide and amino acid identities between the strain SLO/12/06 and the strains SLO/17/04, SLO/872/04, SLO/218/05, SLO/718/05, SLO/802/05 and SLO/75/06 were 99.6% and 98.8 to 100%, respectively.

PPMV-1 isolated from the blackbird has a ^{112}RRQKRF^{117} sequence at the F protein cleavage site (Figure 2).

The strain SLO/12/06 isolated from a blackbird was classified, together with the other PPMV-1 viruses associated with the ongoing panzootic in pigeons, into sublineage 4b of lineage 4 (Figure 1). Strain SLO/12/06 clusters together with PPMV-1 strains isolated from free-living and domestic pigeons in Slovenia between 2001 and 2008, as well as with the European PPMV-1 isolated from pigeons and doves between 1999 and 200l. These strains, together with PPMV-1 strains isolated from other bird species (budgerigar, dove, swan, kestrel and cockatoos), belong to group 4bii of sublineage 4b (Figure 1).

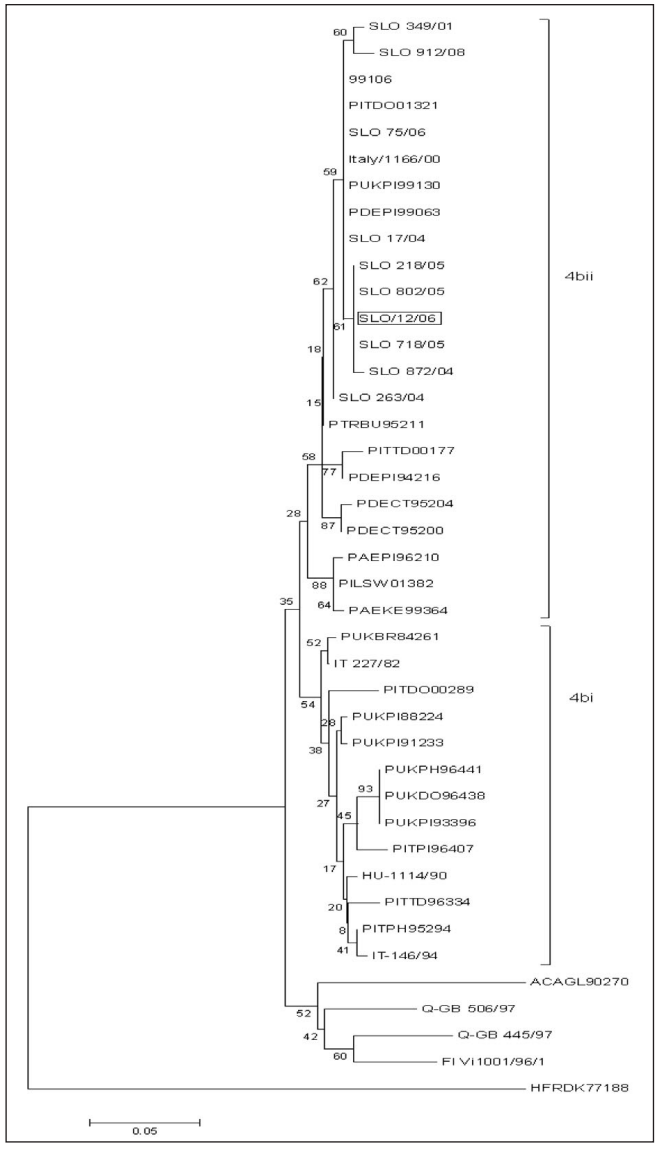

Figure 1: Phylogenetic tree of the region at the 3' end of the fusion protein gene of PPMV-1 viruses isolated from pigeons and other bird species. PPMV-1 strain isolated from the blackbird (framed) cluster together with the strains from group-4bii of sublineage 4b. Phylogenetic analyses were constructed by the neighbor-joining method with the Kimura-2 parameter substitution model and 1000 bootstrap replicates to assign a confidence level to branches. The bootstrap test confidence levels are shown next to the branches

Discussion

An APMV-1 virus (SLO/12/06) was isolated from the cloacal swab of a dead blackbird during the regular monitoring of wild birds for the purpose of early detection of the highly pathogenic avian influenza (HPAI) H5N1 virus. On the basis of the results of molecular characterization of the partial F gene, we can conclude that APMV-1 virus isolated from the blackbird belongs to the pigeon variant of avian paramyxovirus type 1 (PPMV-1) (Figure 1). The strain SLO/12/06 isolated from the blackbird was classified, together with other PPMV-1 viruses associated with the ongoing panzootic in pigeons, into group 4bii sublineage 4b (Figure 1). Group 4bii includes PPMV-1 isolated from pigeons and other bird species from Europe, North America and Asia. Isolates from the group 4bii have been the predominant strains in the latter period of the ongoing panzootic of PPMV-1 viruses in pigeons, with the number of the isolations of viruses from group 4bi diminishing from the late 1980s onwards (13).

The finding that the recently isolated strain from the blackbird, SLO/12/06, is highly related to the strains obtained from domestic pigeons (SLO/17/04 and SLO/872/04) and strains from free-living pigeons (SLO/218/05, SLO/718/05, SLO/802/05 and SLO/75/06) (Figure 1) suggests that the same strains have been circulating between the population of free-living and domestic pigeons and that the blackbird was probably infected from one of them.

PPMV-1 isolated from the blackbird has the ^{112}RRQKRF^{117} sequence at the F protein cleavage site. This sequence, found in the majority of recently isolated PPMV-1 viruses (4, 28), is identical to that associated with highly virulent ND viruses (8). However, this prerequisite for high virulence has not always correlated with the pathogenicity of PPMV-1 strains for chickens (30, 40), although it has been demonstrated that the virulence of the majority of PPMV-1 viruses for chickens is greatly increased following three to four passages through this host (25). The PPMV-1 virus isolated from the blackbird has amino acid sequences associated with virulence at the fusion protein cleavage site and, as such, has to be considered a potential threat for poultry.

To the best of our knowledge, this is the first description of the detection of PPMV-1 virus in a blackbird. This indicates that PPMV-1 viruses may infect this bird species. However, we can not draw any conclusion about the virulence of the PPMV-1 virus and the disease that the virus may cause in these birds, because no post mortem examinations of the dead blackbird were performed.

The incidence of APMV-1 viruses in wild passerine birds was investigated over a three-year period. The results of the study showed that cloacal swabs taken from 598 passerine birds, including 11 blackbirds, between 2004 and 2006, were negative for APMV-1 viruses by virus isolation on embryonated

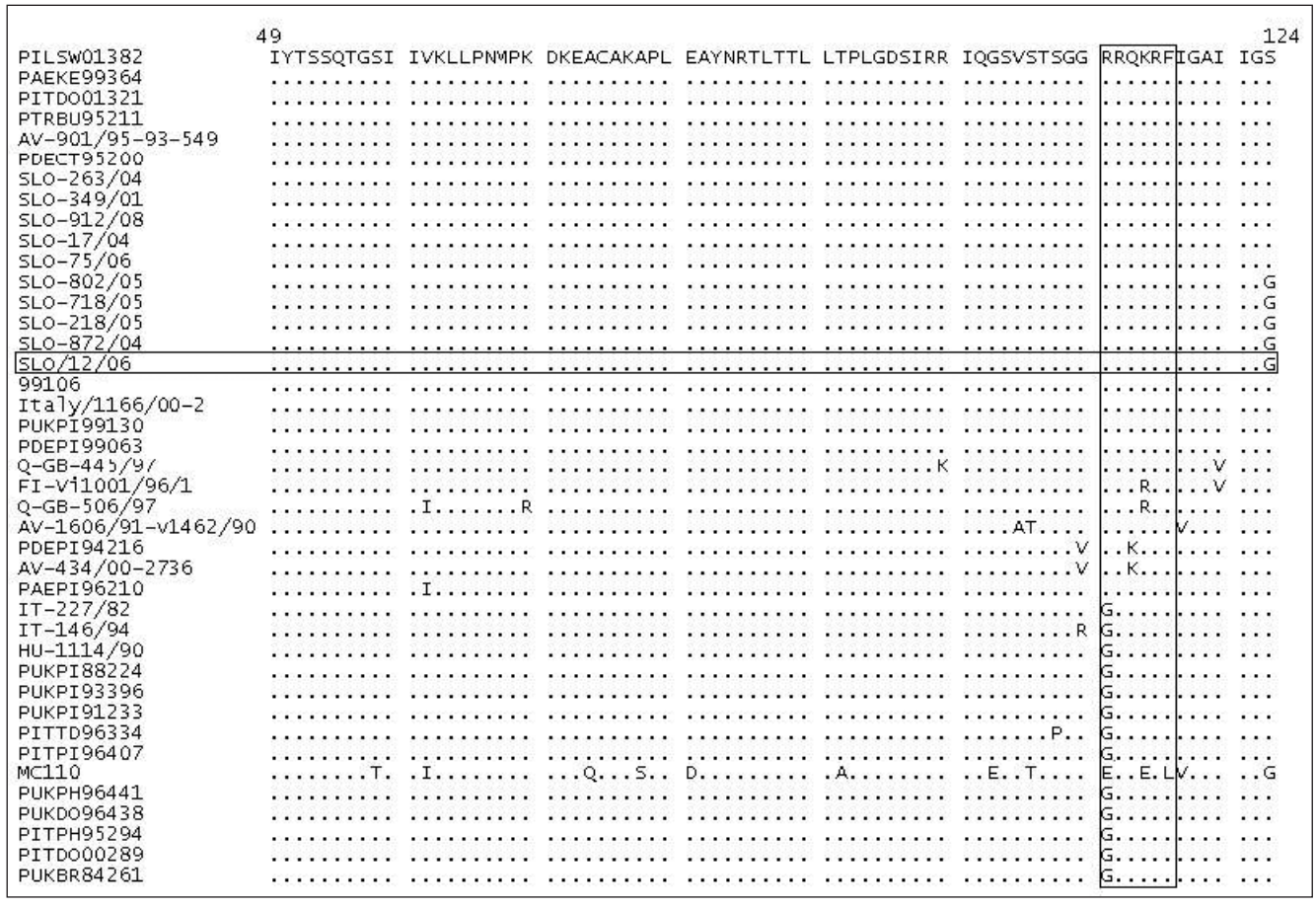

Figure 2: Amino acid sequence alignment of partial PPMV-1 viruses' fusion protein sequences of PPMV-1 isolated from the blackbird and other PPMV-1 strains used in the study. The fusion protein cleavage site sequence from position 112 to 117, considered as a major determinant of strain pathogenicity for poultry, and partial F protein amino acid sequence of PPMV-1 virus isolated from a black bird are boxed

SPF fowl eggs (41). These results, as well as other data from the literature indicate that song birds play only a minor role as a potential disseminator of APMV-1 viruses (35).

Nevertheless, PPMV-1 viruses, which are endemic in the pigeon population in many European countries, can infect robins (33) and blackbirds as reported in the present study. The spread of the virus in the population of passerine birds and the threat that this could represent to these bird species and to poultry production should therefore further investigated.

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IZOLACIJA IN MOLEKULARNA KARAKTERIZACIJA GOLOBJE VARIANTE AVIARNEGA PARAMIKSOVIRUSA TIPA 1 (PPMV-1), UGOTOVLJENEGA PRI KOSU *(TURDUS MERULA)*

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Povzetek: Brisa sapnika in kloake, odvzeta mrtvemu kosu smo preiskali v sklopu povečanega virološkega nadzora prostoživečih ptic, da bi dovolj zgodaj ugotavljali zelo patogen virus aviarne influence H5N1. Iz brisa kloake smo pri 10 dni starih kokošjih embriih brez specifičnih protiteles izolirali golobjo različico aviarnega paramiksovirusa tipa 1 (PPMV-1). Molekularno karakterizacijo izoliranega virusa smo izvedli z metodo obratnega prepisa in verižne reakcije s polimerazo ter določanjem zaporedja nukleotidov na območju gena za fuzijski (F) virusni protein, vključno z območjem, ki določa mesto cepitve virusnega proteina F. Zaporedji nukleotidov in aminokislin območja na genu za protein F pri virusu PPMV-1, ugotovljenem pri kosu, sta bili najbolj podobni zaporedjem nukleotidov in aminokislin pri virusih PPMV-1, ugotovljenih pri prosto živečih in domačih golobih med leti 2004 in 2006 v Sloveniji. S filogenetsko analizo smo virus uvrstili v linijo 4, podlinijo 4b virusov PPMV-1. Zaporedje aminokislin na mestu cepitve proteina F pri virusu PPMV-1, ugotovljenem pri kosu, je bilo ^{112}RRQKRF^{117}. Ugotovljeno zaporedje aminokislin je značilno za virulentne aviarne paramiksoviruse tipa 1 (viruse newcastelske bolezni). Glede na nam dosegljive podatke menimo, da gre za prvi opis izolacije in molekularne karakterizacije virusa PPMV-1, ugotovljenega pri kosu.

Ključne besede: kos *(Turdus merula)*; aviarni paramyxovirus tipa 1; NDV; zaporedja nukleotidov in aminokislin gena za protein F; filogenetska analiza