The first confirmed cases of pigeon rotavirus A (RVA) infection in domestic pigeons (Columba livia) in Poland

Krzysztof Adamczyk¹, Dennis Rubbenstroth², Aleksandra Ledwoń¹, Rafał Sapierzyński¹, Piotr Szeleszczuk¹

¹Department of Pathology and Veterinary Diagnostics, Warsaw University of Life Sciences, 02-776 Warszawa, Poland
²Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, 17493 Greifswald - Insel Riems, Germany
krzysztof_adamczyk@sggw.edu.pl

Abstract

Introduction: Although the presence of rotaviruses in pigeon samples has been reported since the 1980s, its importance as an aetiopathogenic agent of the “classical” young pigeon disease (YPD) was not proven until 2020, when the Henle–Koch postulates were confirmed for pigeon-type rotavirus A (RVA) genotype G18P(17). Material and Methods: From 2011 to 2020, archived liver samples from 117 pigeons submitted by 74 individual lofts were tested for the presence of pigeon-type RVA using a VP6-specific RT-qPCR test. For four positive racing pigeons, a more detailed necropsy and histopathological analysis was performed. Results: Indicators of an acute RVA infection were found in 24 out of 117 (20.5%) samples tested, the earliest in 2014. Necropsies of the four selected RVA-positive pigeons showed changes mainly in the liver, spleen and kidneys similar to those described by other researchers. The histopathological examination revealed mainly hyperaemia and necrosis in the liver, as well as mononuclear cell infiltrates in these organs. Conclusion: Pigeon-type RVA is also a cause of YPD in Poland and is a serious challenge for racing pigeon breeders and veterinarians, especially during the training and flights of young pigeons.

Keywords: pigeon-type rotavirus A, racing pigeon, young pigeon disease, pigeon viral diseases.

Introduction

Young pigeon disease (YPD) is a common problem in the breeding of domestic pigeons (Columba livia) and has been known for more than three decades (6, 16, 21). Various pathogens, including pigeon adenoviruses, pigeon circovirus 1 and Escherichia coli had been suspected to be involved (7, 21, 25) prior to the 2020 fulfilment of the Henle–Koch postulates and confirmation of a pigeon-associated clade of rotavirus A genotype G18P(17) as the causative agent of “classical” YPD (16, 23). Rotavirus A (RVA) is a double-stranded RNA virus in the Rotavirus genus and Reoviridae family. Although rotavirus infections including RVA G18P(17) had been diagnosed much earlier in pigeons, they were not clearly associated with diseases of young pigeons at that time (9, 17, 18, 26). Fatal diseases of juvenile pigeons associated with RVA have been reported in Australia, Europe and the USA (2, 3, 10, 13, 15, 22, 24). In most cases, pigeon-type RVA was able to establish systemic infection and it necrotised birds’ livers (3, 15, 16, 22, 23). Infection with RVA spreads quickly through a loft and typically causes high morbidity up to 100% and mortality ranging from none to more than 50% (3, 13, 15, 16, 22). Usually, the course of disease in affected flocks lasts around one week, and it takes up to three weeks for recovered pigeons to regain weight (23). Clinical manifestations of RVA infection included apathy, anorexia, slimy greenish diarrhoea, vomiting and a congested crop (15, 16, 22, 23). In contrast, neurological or respiratory signs were only rarely reported (15, 22, 23). Gross lesions observed at necropsy were an enlarged and diffusely mottled liver and a moderately enlarged, pale spleen. The kidneys were usually enlarged and sometimes pale. The crop was often filled with watery content and the intestine content of some pigeons was green and watery (3, 24).

Poland is one of the world-leading nations in pigeon breeding (8). According to the Federation Colombophile Internationale, based on the number of rings distributed to Polish breeders, around 4 million juvenile racing pigeons are hatched annually by over

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40,000 breeders associated in clubs (8, 20). As for fancy pigeons, about 3,000 breeders are members of the Polish Association of Purebred Pigeon and Small Livestock Breeders, and approximately 200,000 rings were distributed in 2022 (19), but it is suspected that many owners of fancy pigeons are not members of any organisation. In any case, transport to racing competitions and staging exhibitions brings high numbers of juvenile birds into very close direct contact and provides an ideal environment for the spread of pathogens, including pigeon-type RVA (5, 13).

The RVA genome is composed of 11 segments of double-stranded RNA, which encode six structural proteins (VP1 to VP4, VP6, and VP7) and six nonstructural proteins (NSP1 to NSP6) (12, 14). The PCR assay targeting VP6 was shown to be the most sensitive qualitative reverse transcription PCR (RT-qPCR) assay because it produced lower values of the cycle of quantification (Cq); therefore, it was used in this study (15, 22). Here the first reported clinical cases of pigeon-type RVA infection are described which were found in young domestic pigeons in Poland, and pigeon-type RVA sequences determined from archived samples from 2011–2020 are analysed phylogenetically.

**Material and Methods**

**Origin of samples.** From 2011 to 2020, archived liver samples from 117 pigeons (66 fancy and 51 racing birds) originating from 74 individual lofts were tested for the presence of pigeon-type RVA. Six of these samples were formalin-fixed paraffin-embedded (FFPE) material, and the remaining samples had been frozen when fresh. They originated mainly from the Mazovia voivodeship (n = 89), but also from Warmia and Mazury (n = 9), Pomerania (n = 4), Lódź (n = 2), Silesia (n = 1) and Greater Poland voivodeships (n = 1). The region of origin was unknown for eleven individuals (Supplementary Table 1). A more detailed necropsy was performed on four pigeons from three lofts and a histopathological examination was carried out on three pigeons from these lofts. Clinical data was also provided by the breeders of these four pigeons. They were approximately four-month-old racing pigeons that were delivered for necropsy in July 2017 and August 2018. They originated from three different lofts from the Lódź, Silesia and Mazovia voivodeships.

**Necropsy and histopathology.** Necropsy and sample collection were performed for all pigeons according to standard avian necropsy protocols in the Department of Pathology and Veterinary Diagnostics in the Institute of Veterinary Medicine of the University of Life Sciences in Warsaw. Livers were collected in sterile tubes and stored at −20°C until extraction of nucleic acids was carried out. For histopathological examination, samples of the heart, liver, kidney, spleen, duodenum and pancreas were collected during necropsy from three pigeons submitted in 2017 and 2018 from three different outbreaks. They were fixed in 10% buffered formalin and embedded in paraffin following standard protocols. The paraffin blocks were cut into 5 µm sections, stained with haematoxylin and eosin and examined using light microscopy.

**Extraction of RNA and detection of RVA-specific RNA.** Total RNA was extracted from comparable pea-sized sections of frozen liver samples using a NucleoMag VET kit (Macherey-Nagel, Düren, Germany) with a KingFisher Flex Purification System (Thermo Fisher Scientific, Waltham, MA, USA). Ribonucleic acid from FFPE samples was extracted using a mirNeasy FFPE Kit (Qiagen, Hilden, Germany). All procedures were performed according to the manufacturers’ instructions. A defined number of copies of in vitro-transcribed RNA of the egfp enhanced green fluorescent protein gene was added to each sample during RNA extraction as an internal control to confirm the efficiency of RNA extraction and the RT-qPCR reaction (11). Screening for pigeon RVA was performed with a previously published VP6-specific RT-qPCR assay (forward primer: CoRVA_VP6_868+, 5’-GCCCGYAATTCTGATGATAC−3’; reverse primer: CoRVA_VP6_943−, 5’-GCGCCTGATGATAC−3’; TaqMan probe: CoRVA_VP6_898_P, 6-AM-5’-TTCCACTTGTGTTGCCGACC−3’-BHQ1) (22) with AgPath-ID One-Step RT-PCR reagents (Thermo Fisher Scientific). Briefly, 5 µL of extracted RNA was mixed with 2 µL of primer mix (containing each primer at a concentration of 5 µM) and denatured at 95°C for 5 min. Subsequently, 18 µL of master mix containing 12.5 µL of AgPath 2× buffer, 2 µL of AgPath enzyme mix (reverse transcriptase and Taq polymerase), the RVA-specific probe at a final concentration of 0.12 µM and primers and probes for the detection of egfp RNA was added and the reaction was performed with the following cycler setup: 45°C for 10 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Standard preparations of cell culture supernatant and extracted RNA from isolates DR-7 and DR-5 (22), respectively, served as respective positive controls of RNA extraction and RT-qPCR. Cycle of quantification values were calibrated by adjusting the threshold to set the Cq values of the two positive controls to constant values (+/− 0.5) in each RT-qPCR run. Values lower than 30 were considered as indicative of an acute RVA infection (22, 23).

**RVA sequencing and phylogenetic analysis.** Partial VP6 gene sequences were generated for all RVA-positive pigeons with a Cq value <30. A 438-base pair (bp) fragment of the VP6 segment was amplified by conventional RT-PCR using AvRVA_VP6_243+ (5’-GAGGCGAATTACGTTGAGAATG−3’) and CoRVA_VP6_680− (5’-TCTAAYACTTTCGAGTTGCC−3’) primers (22). Following gel electrophoresis, products of the expected length were extracted using Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). Sanger sequencing was performed by Eurofins Genomics (Ebersberg, Germany). Raw sequences were trimmed for quality and primer sequences to achieve
a final sequence of 392 bp. Sequences were submitted to GenBank under accession numbers OR400329 to OR400352.

Phylogenetic analysis was performed for all partial VP6 sequences generated during this study together with avian RVA VP6 sequences derived from GenBank. Following nucleotide sequence alignment using MUSCLE, a phylogenetic tree was built using the neighbour-joining algorithm and Jukes–Cantor distance model in Geneious Prime 2021.0.1 (Biomatters Ltd., Auckland, New Zealand). Avian RVA VP6 sequences of genotypes I11 and I21 were used to root the tree.

Results

Detection of pigeon-type RVA-specific RNA. Using a VP6-specific RT-qPCR test, different levels of pigeon-type RVA RNA could be detected in 57 out of 117 samples (48.7%) (Fig. 1A, Supplementary Table 1). Based on previous experiments, only calibrated Cq values below 30 were considered indicative of an acute or recent RVA infection, whereas calibrated Cq values above 30 were considered as possibly indicating remnants of a past infection (22, 23). Such positive results were obtained for 24 out of 117 (20.5%) tested samples. Positive test results were obtained for 12 out of 51 (23.5%) samples from racing pigeons and 12 out of 66 (18.2%) samples from fancy pigeons (Fig. 1A). The positive pigeons originated from 19 out of the 74 tested lofts (25.7%). Positive samples were detected in the years 2014, 2015 and 2017 to 2019 (Fig. 1B, Supplementary Table 1). Outbreaks of RVA infection in racing pigeons were detected only between May and September, with most positive samples dating to August (Fig. 1C). The samples from fancy pigeons which were RVA positive were more evenly distributed over the year, with most positive samples submitted in December (Fig. 1C).

Necropsy findings. Detailed necropsy findings were available for four RVA-positive pigeons from three different outbreaks: birds A1 and A2 from the Łódź voivodeship, which were sick in 2018; bird B from the Silesia voivodeship, which was a case from 2018; and bird I from the Mazovia voivodeship, which was infected in 2017. Pigeons A1, A2, B and I had been tested positive for RVA by RT-qPCR. In two of these pigeons, the liver was moderately enlarged and congested (Fig. 2, photographs of pigeons A1 and B), and in the other two it was enlarged and mottled (Fig. 2, photographs of pigeons A2 and I). The kidneys of all four pigeons were enlarged; in one pigeon it was congested (Fig. 3, photograph of pigeon A1), and in two pigeons it was pale (Fig. 3, photographs of pigeons A2 and B). The spleen was pale and slightly or moderately enlarged in two pigeons (Fig. 4, photographs of pigeons B and I) and normal-sized and pale in one pigeon (Fig. 4, photograph of pigeon A1). The presence of food with water and bile was observed in the crop of three pigeons, and in these cases bile was also present in the proventriculus. The content of the intestines was green and mucoidal to watery in consistency. The content of the distal part of the gastrointestinal tract was dark green to brown and mucoidal. The vent and the surrounding feathers of two birds were stained with slimy faeces. In three pigeons, the air sacs showed varying degrees of opacity. Also in three pigeons, extensive areas of myocardial discoloration were found (Fig. 2, photographs of pigeons A2, B and I). The condition of the pigeons ranged from very good in two of the cases to satisfactory.
Fig. 2. Macroscopic changes in the livers and hearts of four rotavirus A-positive pigeons (A1, A2, B and I) originating from three outbreaks (A in the Łódź, B in the Silesia and I in the Mazovia voivodeships). Liver congestion (A1 and B); liver enlargement and mottling and myocardial necrosis (A2 and I).

Fig. 3. Macroscopic changes in the kidneys from three selected rotavirus A-positive pigeons (A1, A2 and B) originating from three outbreaks (A in the Łódź, B in the Silesia and I in the Mazovia voivodeships). Kidney oedema (A1, A2 and B); simultaneous pallor (B).

Fig. 4. Macroscopic changes in the spleens of three selected rotavirus A-positive pigeons (A1, B, and I) from three outbreaks (A in the Łódź, B in the Silesia and I in the Mazovia voivodeships). Spleens appearing pale and slightly to moderately enlarged (A1, B and I).
Fig. 5. Microscopic lesions stained with haematoxylin and eosin in the livers of two selected rotavirus A-infected pigeons (A and B) from two outbreaks (A in the Łódź and B in the Silesia voivodeships). Severe congestion, and focal, mainly perivascular mononuclear inflammatory infiltrate (white arrows), consisting of lymphocytes, plasma cells and histiocytes, degenerative and necrotic changes of hepatocytes (black arrows) – magnification 100× (A); severe congestion, haemorrhaging, massive haemorrhagic necrosis of hepatic parenchyma, and perivascular inflammatory infiltrate (white arrows) – magnification 40× (Ba); severe congestion, haemorrhaging and perivascular mononuclear inflammatory infiltrate (white arrows), consisting of lymphocytes, plasma cells and histiocytes – magnification 200× (Bb).

Fig. 6. Phylogenetic analysis of partial rotavirus A VP6 genotype I4 sequences from pigeons, domestic poultry, and other species. Sequences 392 base pairs long originating from Polish pigeons were analysed together with sequences obtained from GenBank. Bootstrap values above 70 are indicated at major branches. Colours indicate different pigeon-associated subclades as published by Rubbenstroth et al. (22). Subclades are labelled with the year of first reported detection in European pigeon populations or with the non-European country of detection. Sequences generated during this study are in bold.
Histopathology. Microscopic analysis was performed for three RVA-positive pigeons (A1, B and I). Lesions observed in the liver were focal perivascular mononuclear infiltrates (Fig. 5 A1, Ba and Bb), necrosis (Fig. 5 Ba), congestion and haemorrhaging (A1), and steatosis and bile retention (not shown). In the kidneys, congestion, haemorrhaging, heterophilic perivascular infiltrates and accumulation in the epithelium of cells were seen (not shown). In one bird (I) bile in coronal fat tissue, myocardial congestion and focal necrosis were found (not shown).

Phylogenetic analysis of partial VP6 sequences. Partial VP6 sequences were generated for all 24 RVA-positive pigeons. Phylogenetic analysis of partial VP6 sequences together with sequences from pigeons, domestic poultry and other species available in GenBank was performed and revealed all Polish sequences to belong to the pigeon-type RVA VP6 genotype 14 (Fig. 6). Six sequences detected in 2014 and 2015 clustered with sequences detected in Germany in 2010 to 2014 (subclade 2010, according to the nomenclature published by Rubbenstroth et al. (22)), whereas 14 Polish sequences from 2017 to 2019 clustered together with German sequences from the same years (subclade 2017b). Interestingly, a further sequence from 2015 and three sequences detected in 2018 formed two separate branches tentatively labelled subclades 2015b and 2018 (Fig. 6).

Discussion

Pigeon-type RVA was recently demonstrated as the causative agent of “classical” YPD in domestic pigeons. However, while the disease has been reported throughout Europe, data on the occurrence of the virus in Europe has come so far almost exclusively from a single country, Germany (22, 24). Here we confirmed the detection of pigeon-type RVA infections in archived liver samples from Poland from the years 2014 to 2015 and 2017 to 2019.

The racing season of young pigeons in Poland runs from the middle of August to the end of September, while adult pigeon races take place from the end of April to the end of July (20). An additional risk factor, both for racing and fancy pigeons, are pigeon exhibitions, which take place in autumn and winter (19, 24). It is worth noting that RVA-positive samples from racing pigeons were collected in the period from April to September, i.e. during pigeon training and racing. A particularly high number of cases was observed in the summer, when training and flights of young pigeons take place. In fancy pigeons, the period in which positive results were obtained was longer, with the highest number of cases in December, when fancy pigeon exhibitions were held. The cases of RVA infection in racing pigeons described by Blakey et al. (3) occurred from April to November, and this larger time window may be related to differences in racing season lengths between the Polish and Californian, especially for young pigeons, the Californian season having lasted until December (4). The first cases reported in Australia occurred from late May to July, although there have been sporadic cases also in October, December and April, usually after transport with pigeons from other lofts (15). In studies conducted by Hunnam et al. (13), also in Australia, cases of the disease were recorded mainly from December to May depending on the state. In these cases, especially in December, it was not so much the races that contributed to the infection as the purchase of new pigeons and their introduction into the loft (13). It should be noticed that the racing season in Australia runs from the end of May to October (1). Clinical disease induced by RVA is to be expected mainly during the racing and exhibition period, when juvenile pigeons are usually exposed to the pathogen for the first time, although isolated cases may occur in the off-season.

Post-mortem changes in the pigeons described here spanned a relatively wide range in the liver, from only hepatic congestion, through severe enlargement and diffuse mottling, to a complete change in the colour of the organ to yellowish-orange. A slightly narrower range of changes in the liver, from their absence to abnormal enlargement of the organ with darkly mottled congested and friable tissue, was described by Blakey et al. (3). Noteworthily, changes in the myocardium were observed in three pigeons, which had not been described prior to this in association with RVA infection. Opacity of the air sacs was also described in pigeons studied by Blakey et al. (3) and was probably related to aspiration of food into the respiratory system shortly before death (3, 15, 24). Liver necrosis and focal mononuclear cell infiltrates were found in two out of three histopathologically examined pigeons; this symptom was also dominant in all previously described naturally occurring cases (3, 15, 22, 24). It is worth noting that, unlike the pigeons necropsied by other authors, one of the pigeons studied by us also had necrotic changes in the heart muscle.

Pigeon-type RVA RNA was isolated from 48.7% of all samples collected from 2011 to 2020. However, high calibrated Cq values indicating low amounts of viral RNA were detected in many of these samples. Previous studies have shown that low amounts of viral RNA may remain detectable in cloacal swabs and internal organs for several weeks after an RVA outbreak in a pigeon loft (15, 22, 23). Based on this empirical data, we consider the calibrated Cq values of >30 to likely represent remnants of an infection that had occurred several weeks before sampling. Further analysis was focused on potentially acute infections, indicated by the calibrated Cq values of <30 (20.5% of all samples). The earliest of these cases were found in 2014, while in samples collected in the USA from 2000–2018, the presence of RVA was detected in 0.6% of the samples, first from 2001 (3).

Conclusion

Pigeon-type RVA has been causing losses among domestic pigeons since a much earlier period than the
time when it was first described in association with clinical disease (16, 22). Pigeon-type RVA infections are currently a serious problem in pigeon breeding. Fortunately, countermeasures in the form of vaccination have already been established. Both autogenous vaccines and licensed vaccines are successfully used in Europe (16, 24).

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