Received: 24 September 2019 | Revised: 3 December 2019 | Accepted: 15 January 2020

DOI: 10.1111/tbed.13485

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ORIGINAL ARTICLE

Transboundary and Emerging Diseases WILEY

First experimental proof of *Rotavirus A* (RVA) genotype G18P[17] inducing the clinical presentation of 'young pigeon disease syndrome' (YPDS) in domestic pigeons (*Columba livia*)

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Funding information
Horizon 2020 Framework Programme, Grant/Award Number: 643476

Abstract

Young pigeon disease syndrome (YPDS) is characterized as a seasonally occurring, acute and primarily enteric medical condition of mainly juvenile domestic pigeons (*Columba livia*) with highly variable mortality reaching more than 50%. Although the syndrome has been known in Europe for almost three decades, its aetiology remains largely obscure. Recently, a previously unknown pigeon-associated clade of *Rotavirus A* (RVA) genotype G18P[17] was detected in Europe and Australia in association with fatal diseases resembling YPDS. Here we show for the first time, that peroral inoculation of healthy juvenile homing pigeons with two genetically different cell culture isolates of RVA G18P[17] ($10^{6.3}$ foci-forming units per bird) induces an acute and self-limiting YPDS-like disease in all infected birds. Clinical signs included regurgitation, diarrhoea, congested crops, anorexia and weight loss, as described for naturally RVA-infected pigeons. In agreement with the original outbreaks, RVA isolate DR-7 induced more pronounced clinical signs as compared to isolate DR-5, indicating strain-dependent virulence factors to contribute to variable disease outcomes observed in the field. All inoculated birds developed rotavirus-reactive antibodies starting at seven days after inoculation. High levels of viral RNA and infectious virus were detectable in cloacal swabs and faecal samples already three days after inoculation. While shedding of infectious virus subsided within few days, moderate viral RNA levels were still detectable in cloacal swabs, faeces, and tissue samples at the end of the experiment three weeks after inoculation. Histopathological analysis at this time point revealed inflammatory lesions in spleens and livers of pigeons from both infected groups. In summary, we fulfilled Henle-Koch's postulates and confirmed RVA G18P[17] as a primary cause of YPDS-like diseases in domestic pigeons. By establishing an infection model, we provide a crucial tool for future research, such as identification of transmission routes and establishing vaccination regimes.

KEYWORDS

aetiology, domestic pigeons, experimental infection, Rotavirus A, young pigeon disease syndrome

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1 | INTRODUCTION

Members of the viral species *Rotavirus A* (RVA; genus *Rotavirus*, family *Reoviridae*) are non-enveloped viruses with a double-stranded RNA genome composed of 11 segments. They are known as causative agents of acute intestinal disorders of mainly juvenile individuals of a broad range of different species, including humans, domestic mammals and birds (Badur et al., 2019; Dhama, Chauhan, Mahendran, & Malik, 2009; Dhama et al., 2015; Martella, Banyai, Matthijnssens, Buonavoglia, & Ciarlet, 2010). While most RVA infections remain largely restricted to the intestinal tract, recent evidence suggests the occurrence of systemic infections particularly in young children (Gomez-Rial et al., 2019; Martella et al., 2010; Ramig, 2004; Rivero-Calle, Gomez-Rial, & Martinon-Torres, 2016).

In domestic pigeons (*Columba livia*), RVA had been sporadically detected in healthy and diseased individuals (Gough, Cox, & Devoy, 1992; Minamoto, Oki, Tomita, Kinjo, & Suzuki, 1988; Pauly et al., 2017). However, its pathogenic potential in this species remained largely unknown until a pigeon-associated clade of RVA genotype G18P[17] was described recently in association with fatal diseases in Europe and Australia (Hunnam, Sloan, McCowan, Glass, & Walker, 2019; McCowan et al., 2018; Rubbenstroth et al., 2019). Remarkably, these viruses were found to establish systemic infections with high viral loads particularly in the liver. Consequently, hepatic necrosis is usually the most prominent lesion observed during acute infection (McCowan et al., 2018; Rubbenstroth et al., 2019). Typical clinical signs include apathy, anorexia, slimy diarrhoea, vomiting and congested crops, whereas neurologic and respiratory signs or fibrinous lesions are rarely observed. In affected flocks, the disease usually subsides within approximately a week and the mortality may range from none to more than 50% (Gough et al., 1992; Hunnam et al., 2019; McCowan et al., 2018; Rubbenstroth et al., 2019). Factors influencing the outcome of the infection are poorly characterized, but may include strain-dependent virulence factors, genetic differences in the host, age, maternally derived immunity as well as the presence of other infectious or non-infectious factors (Rubbenstroth et al., 2019).

In European domestic pigeon populations, natural RVA infection was found to be closely associated with a widely distributed medical condition known as 'young pigeon disease syndrome' (YPDS) (Rubbenstroth et al., 2019). YPDS has been known for almost 30 years and is characterized by a seasonal occurrence mainly in juvenile birds during the racing season of homing pigeons in summer and after exhibitions of ornamental pigeons during winter. Its aetiology remains poorly understood, and until recently, it has not been connected to a particular primary cause. Instead, a multifactorial aetiology has been discussed, potentially involving various pathogens such as pigeon circovirus 1 (PiCV-1), several avian adenoviruses, *Escherichia coli*, *Spironucleus* spp. and others. However, a clear association could not be established for any of these candidates and experimental infections were either not possible due to the lack of viral isolates or did not result in reproduction of the disease (Raue et al., 2005; Schmidt et al., 2008; Stenzel, Pestka, Tykalowski, Smialek, & Koncicki, 2012; Teske et al., 2017; Zhang, Dai, Wang, & Dai, 2015).

Here we report the experimental infection of healthy juvenile domestic pigeons with two different RVA G18P[17] isolates originating from YPDS-like outbreaks in Germany. Inoculated animals developed an acute, self-limiting disease matching the clinical presentation observed during YPDS and natural RVA infections, thereby confirming for the first time the role of pigeon-associated RVA G18P[17] as primary pathogens of domestic pigeons.

2 | MATERIAL AND METHODS

2.1 | Virus isolates and virus preparation

Two genetically different pigeon RVA isolates of genotype G18P[17] were used in this study. Isolate DR-5, representing VP6 sublineage 2015 (Rubbenstroth et al., 2019), originated from a pooled faecal sample collected from a YPDS outbreak in a homing pigeon flock with 2.5% mortality (Germany, July 2016). Isolate DR-7, representing VP6 sublineage 2017a (Rubbenstroth et al., 2019) was isolated from the liver of a racing pigeon with severe hepatic necrosis that had died during an outbreak with 12.5% mortality (Germany, June 2017).

The viruses were isolated, passaged and titrated using MA-104 African green monkey kidney cells as described previously (Rubbenstroth et al., 2019). Virus inoculum of both viruses was prepared after three cell culture passages.

The sequence identity of both virus preparations to the respective original sample, as well as the absence of contaminating pathogens, was confirmed by metagenomics analysis employing previously published high-throughput sequencing procedures with subsequent bioinformatics analysis using RIEMS (Scheuch, Hoper, & Beer, 2015; Wylezich, Papa, Beer, & Höper, 2018).

2.2 | Experimental animals and experimental design

In October 2018, 20 two-month-old racing pigeons were purchased from a private breeder, whose flock is under regular veterinary surveillance, including regular vaccination against pigeon-type paramyxovirus 1 (PPMV-1), *Salmonella* Typhimurium and pigeon poxvirus. Yearly, RVA outbreaks had been detected in juvenile pigeons of the flock from 2012 to 2017, but not in 2018. RVA, *Salmonella* spp., *Eimeria* spp. and helminths were not detectable in pooled faecal samples collected from the experimental birds before the beginning of the experiment. The birds were housed in a biosecurity level (BSL) 2 isolation unit, provided with drinking water and grit ad libitum and offered 30 to 35g commercial pigeon feed per bird and day.

Following four weeks of adaptation to the experimental conditions, the birds were divided into three separately housed groups at the age of three months. Group A (four birds) remained uninfected, while groups B and C (eight birds, each) received $10^{6.3}$ focus-forming units (ffu) of RVA isolate DR-5 or DR-7, respectively. Each bird was inoculated with 2 ml virus preparation directly into the crop using a buttoned cannula. Clinical signs and feed uptake were recorded daily

throughout the experiment. The body weight was determined daily during the phase of acute clinical disease and at three to four day intervals during the remaining experimental period. Pooled faecal samples and drinking water from each group and two cloacal swabs from each bird were collected at two to four day intervals. One of each pair of swabs was stored dry for RNA extraction and detection of viral RNA by RT-qPCR, while the other swab was transferred immediately to 0.5 ml serum-free cell culture medium for titration of infectious virus. Serum samples were collected at weekly intervals. At day 21 post-inoculation (p.i.), all birds were euthanized and tissue samples were collected for histopathology, viral RNA detection by RT-qPCR and virus titration. All samples were stored at −80°C until further analysis.

2.3 | Recording of clinical scores and disease parameters

Pigeons were examined at least twice daily throughout the experiment. Clinical scores were recorded in the morning by the same researcher with experience in pigeon husbandry and experimental infection of avian species (D.R.). Total scores (0–13) consisted of five subcategories: general condition (score 0–3), feed intake (0–3), regurgitation (0–1), palpation of the crop (0–3) and body condition (0–3). A detailed description of the scoring system is provided in Table S1.

For scoring, the behaviour of the birds was observed silently for few minutes after entering the room in the morning, before all birds were caught for palpation of crop and body condition, determination of body weight and eventually sample collection. During the absence of the birds, the aviary was checked for diarrhoea and regurgitated seeds. After release of the birds, fresh drinking water and a defined amount of pigeon feed (30–35 g per bird) were offered and the feed intake behaviour was observed. In the afternoon, the remaining feed was completely removed from the aviary and subsequently weighed to calculate the daily feed intake of the group.

2.4 | Detection of viral nucleic acids of RVA, pigeon circovirus 1 (PiCV-1), columbid herpesvirus 1 (CoHV-1) and avian adenoviruses by PCR assays

Viral nucleic acids were extracted from cloacal swabs, faecal pools, drinking water and tissue samples using the NucleoMag VET kit (Macherey-Nagel) with a KingFisher Flex Purification System (Thermo Fisher Scientific) according to the manufacturers' instructions. A defined copy number of in vitro-transcribed RNA of the eGFP gene was added to each sample before RNA extraction as an internal control (Hoffmann, Depner, Schirrmeier, & Beer, 2006). Screening for pigeon RVA was performed by a previously published VP6-specific RT-qPCR assay (forward primer: CoRVA_VP6_868+, 5′-GCCCGYAATTTCGATTCAATACG-3′; reverse primer: CoRVA_VP6_943-, 5′-GTGCTGCYACTCCAGGTGTCAT-3′; TaqMan probe: CoRVA_VP6_898_P, 6FAM-5′-TTCCAACTTGTTAGGCCRCCAA-3′-BHQ1) (Rubbenstroth et al., 2019) with AgPath-ID One-Step RT-PCR reagents (Thermo Fisher Scientific). Briefly, 5 µl extracted RNA was mixed with 2 µl primer mix (containing each primer at a concentration of 5 µM) and denatured at 95°C for 5 min. Subsequently, 18 µl master mix, containing the probe (final concentration: 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, 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Standard preparations of DR-7 cell culture supernatant and DR-5 RNA serving as positive controls of RNA extraction and RT-qPCR were used for calibration of cycle of quantification (Cq) values in each RT-qPCR analysis.

Nucleic acids extracted from cloacal bursa collected at necropsy at day 21 p.i. were tested for pigeon circovirus 1 (PiCV-1) DNA using two previously described PCR assays (Duchatel, Todd, Willeman, & Losson, 2009; Freick, Muller, & Raue, 2008). A conventional PCR targeting the polymerase gene of columbid herpesvirus 1 (CoHV-1) was used for detection of CoHV-1 DNA in liver samples collected at necropsy (Freick et al., 2008). Furthermore, pooled faecal samples collected from each group on days 0, 7, 14 and 21 p.i. were screened for adenoviruses using PCR assays designed for the detection of pigeon adenovirus 1 (PiAdV-1), PiAdV-2a, PiAdV-2b and a broad range of avian adenoviruses (Raue, Hafez, & Hess, 2002; Schrenzel et al., 2005; Teske et al., 2017).

2.5 | Virus titration

Titration of infectious virus was performed following previously described procedures (Rubbenstroth et al., 2019). Homogenates of 10% organ tissue or pooled faecal samples in phosphate-buffered saline (PBS) were mixed with 200 µl chloroform per 1 ml sample preparation, whereas cloacal swabs in 500 µl serum-free cell culture medium were thoroughly vortexed and mixed with 100 µl chloroform. After centrifugation at 6,000 g for 10 min, the aqueous phase was collected and subjected to treatment with 20 µg/ml TPCK-treated trypsin (Sigma) for 1 hr at 37°C. Subsequently, 96-well plates with nearly confluent MA-104 cell layers were inoculated with 10-fold dilution series of trypsin-treated samples in serum-free medium. After one-hour incubation at 37°C, inocula were replaced by serum-free medium with 1.5% Avicel (Sigma-Aldrich), 0.35% bovine serum albumin (BSA) and 1 µg/µl trypsin. Cells were fixed with 4% paraformaldehyde following incubation for three days. RVA-infected cell foci were visualized by immunofluorescence staining with polyclonal goat anti-bovine RVA VP6 (Meridian Life Science) and donkey anti-goat-IgG-Alexa 488 (Jackson Immunoresearch). Titres were calculated as ffu per ml supernatant.

2.6 | Antibody detection by indirect immunofluorescence assay (iIFA)

RVA-reactive antibodies were detected by indirect immunofluorescence assay on RVA-infected MA-104 cells. For preparation of

detection cells, confluent cell layers in 96-well plates were washed twice with PBS and subsequently inoculated with 10^3 ffu per well of isolate DR-7 (equalling a multiplicity of infection of 0.05) in infection medium (serum-free medium with 0.35% BSA and 1 µg/µl trypsin). Wells receiving virus-free infection medium served as negative controls. Following incubation at 37°C and 5% CO_2 for 24 hr, the cells were fixed with 4% paraformaldehyde and subsequently permeabilised with 0.5% Triton X-100. Twofold dilution series of serum samples were prepared in Tris-buffered saline with Tween 20, pH 8.0 (T9039, Sigma-Aldrich), and 50 µl of each dilution were added in parallel to RVA-positive and RVA-negative wells. After incubation for 60 min, the plates were washed three times with PBS and incubated for another 60 min with rabbit anti-pigeon IgG-FITC conjugate (Nordic Immunology) diluted 200-fold in T9039 buffer. After a final washing cycle, the wells were analysed by fluorescence microscopy. For each serum dilution, RVA-positive and RVA-negative wells were compared. Wells were considered positive, if the expected approximately 30% RVA-positive cells could be distinguished from the background staining of uninfected cells in the same well and in the corresponding RVA-negative control well by a granular cytoplasmic signal. All tests were performed with a minimal dilution factor of 20. Samples without detection of specific signal were assigned a titre of < 20. Direct comparisons of antibody titres achieved by using detection cells infected with RVA isolates DR-5 or DR-7 did not reveal any effect of the employed virus (data not shown).

2.7 | Histopathological analysis

Necropsy was performed according to internal standard guidelines under BSL-2 conditions. Specimen from brain, heart, lung, spleen, liver, crop, duodenum, pancreas, bursa and kidney were fixed in 4% neutral-buffered formaldehyde for more than two weeks, dehydrated, embedded in paraffin wax and cut at 4 µm thickness. Haematoxylin–eosin (HE)-stained sections were examined for histopathological lesions using an Olympus CH2 microscope equipped with 4x-, 10x- and 40x-ED-achromate objectives. The grade of typical follicular hyperplasia within spleens and the formation of periportal lymphoid nodules within livers was scored using a semi-quantitative scale as follows: 0 = not obvious; 1 = mild; 2 = moderate; 3 = severe.

2.8 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.1.1 software (Graphpad Software, Inc). Unpaired Student's *t* test was used for group-wise comparisons of body and liver weights of the RVA-inoculated groups B and C with the uninfected group A and for comparison of viral RNA levels, infectious virus titres and rotavirus-reactive antibody titres of the infected groups B and C. Mann–Whitney test was used to compare histopathological scores of groups B and C to those of group A and to compare clinical scores of groups B and C with each other. *P* values below .05 are considered to indicate significant differences between groups.

3 | RESULTS

3.1 | RVA inoculation induces YPDS-like clinical disease in juvenile pigeons

Birds of both RVA-inoculated groups developed clinical signs resembling YPDS beginning at day two to three after infection. Starting with reduced appetite and vomiting of individual birds at day 2 p.i., all birds developed slight to moderate apathy (score 1) and up to complete anorexia (score 2–3) within the next two days. All birds of group C and the majority of birds of group B showed crops filled with seeds (score 1–2) from day 4 to 6 p.i. The body condition was reduced (score 1–2) in three birds of group B and six birds of group C starting at days 3–6 p.i. (data not shown). Total clinical scores were significantly higher in group C as compared to group B at days 3–5 p.i. ($p < .05$). They peaked at day 4 p.i. in both infected groups and declined thereafter. After day 10 p.i., all birds had clinically recovered with the exception of three birds of group C still exhibiting reduced body condition until days 17–21 p.i. (Figure 1a). In line with the observed reduced appetite and anorexia, feed intake in both infected groups was reduced from approximately 20 g per bird and day before the experiment to 12.3 and 2.0 g at day 4 p.i. in group B and C, respectively. After clinical recovery, feed intake of group C steadily increased, finally exceeding 30 g per bird and day until the end of the experiment (Figure 1b). While the mean body weight of group B did not differ markedly from the uninfected control group A, birds of group C showed significantly reduced weight from day 6 to 14 p.i. (unpaired Student's *t* test; $p < .05$) and reached the level of the other groups only at the end of the experiment at day 21 p.i. (Figure 1c).

Overall, clinical signs occurred earlier, showed more pronounced peaks and lasted longer in group C as compared to group B for all recorded parameters. No clinical signs were recorded in the uninfected group A throughout the experiment (Figure 1).

3.2 | RVA-inoculated pigeons are shedding viral RNA and infectious virus

Viral RNA and infectious virus were detected in cloacal swabs collected from all inoculated pigeons as well as in pooled faecal samples from both infected groups, but not in samples collected from the uninfected group A (Figure 2). Highest viral RNA levels in cloacal swabs were detectable already at day 3 p.i. in most birds of group C (Figure 2c), whereas significantly lower levels were detected in group B at this time point (Student's *t* test; $p = .0354$) and peak levels were delayed by two to four days in four birds of this

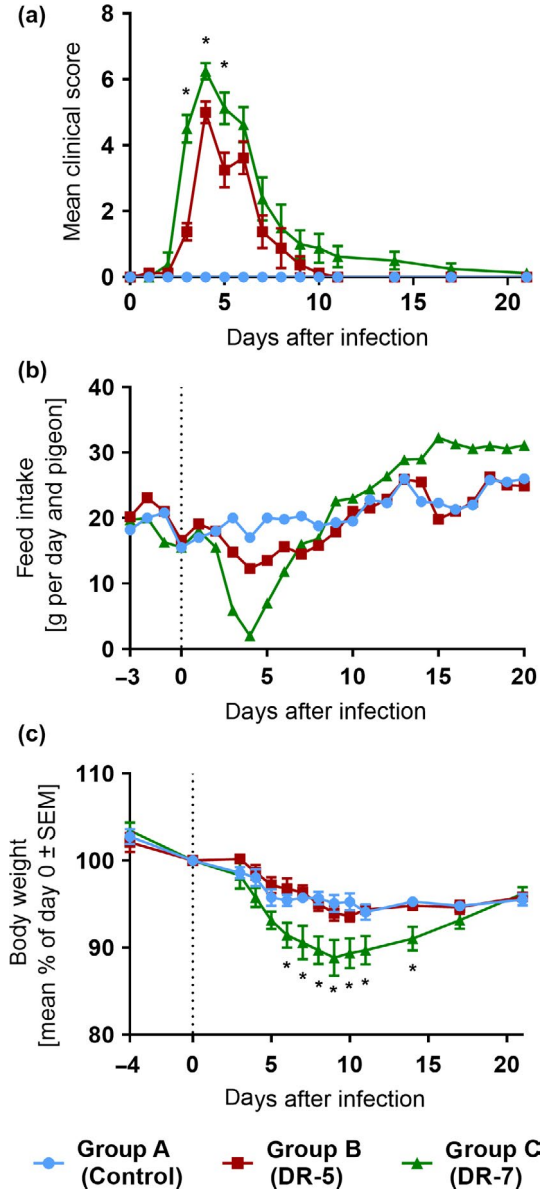

FIGURE 1 Inoculation with pigeon-associated RVA causes disease in juvenile pigeons. (a) Mean clinical score per group. (b) Calculated daily feed intake per bird. (c) Mean body weight relative to day 0 after RVA inoculation. Asterisks indicate significantly different clinical scores between group B and C (panel a; Mann–Whitney test; $p < .05$) or significant differences in body weights of groups B and C as compared to the uninfected control group A (panel c; unpaired Student's *t* test; $p < .05$)

group (Figure 2b). RNA detection decreased rapidly thereafter before keeping steady levels of approximately Cq 25 to 33 until the end of the experiment in both groups (Figure 2b,c). Infectious virus was detectable only at days 3 to 7 p.i. in both groups and mainly in samples with high RNA levels detected by RT-qPCR, as indicated by Cq values ≤ 20 (Figure 2d,e). In line with viral RNA detection, shedding of infectious virus was slightly delayed and prolonged in four birds of group B (Figure 2d). Viral RNA and infection titres in pooled faecal samples largely mirrored the results of cloacal swabs from individual birds (Figure 2f,g), whereas only low RNA levels (Figure 2h) but no infectious virus were detected in drinking water (data not shown).

DNA of pigeon adenoviruses or other aviadenoviruses was not detected in any tested pooled faecal sample collected from the three groups throughout the experiment (data not shown).

3.3 | RVA-inoculated pigeons develop high levels of RVA-reactive antibodies

Detection of RVA-reactive antibodies revealed weak reactivity (iIFA titres 20–40) in about half of the experimental birds at the beginning of the experiment, possibly representing remnants of maternally derived antibodies (Figure 3). After RVA inoculation, the antibody titres increased rapidly in both infected groups, reaching titres of up to 20,000 already at day 7 p.i. and rising further to up to 160,000 until day 21 p.i. (Figure 3). In group B, comparably low titres (320–640) were detected at day 7 p.i. in three birds that had also exhibited delayed viral shedding (Figures 2b,d and 3). At days 14 and 21 p.i., antibody titres in group C were slightly, but significantly higher as compared to group B (Student's *t* test; $p = .0007$ and $p = .0005$, respectively). An increase in RVA-reactive antibodies was not observed in the uninfected group A (Figure 3).

3.4 | Hepatic and splenic lesions are detectable at 21 days after inoculation

At necropsy after 21 days p.i., four out of eight birds of each infected group showed a mild hyperplasia of the spleen. Although the body weight did not differ between the three groups at the end of the experiment (Figure 1c), the relative liver weight was significantly increased in group C as compared to group A ($p < .05$) and the same tendency was observed for group B (Figure 4a). Otherwise, no macroscopic alterations were observed.

Histopathology revealed a mild to severe, multifocal, follicular hyperplasia within the splenic white pulp of all infected pigeons (Figure 5c,e). Scores were significantly increased in both inoculated groups as compared to group A ($p < .05$; Figure 4b). While no or only mild, oligo- to multifocal, periportal, nodular lymphoid aggregates were observable in the livers of most control pigeons (Figure 5b), significantly more abundant and larger lymphoid nodules were detectable in group C ($p < .05$; Figures 4c and 5f). The hepatic lymphoid nodules in groups B and C were occasionally accompanied by adjacent, indistinct, parenchymal infiltrates of lymphocytes and macrophages, occasionally containing intra-histiocytic, intra-cytoplasmic, yellow-brown pigment granules (Berlin blue-positive hemosiderin), interpreted as a subacute, reactive, phagocytic response to a possible, previous degeneration and necrosis of hepatic parenchyma. In contrast to liver and spleen, the grade of lymphoid nodule formation in the pancreas did not differ significantly between the three groups (Figure 4d). No systematic histopathological differences were observed for any other analysed tissues.

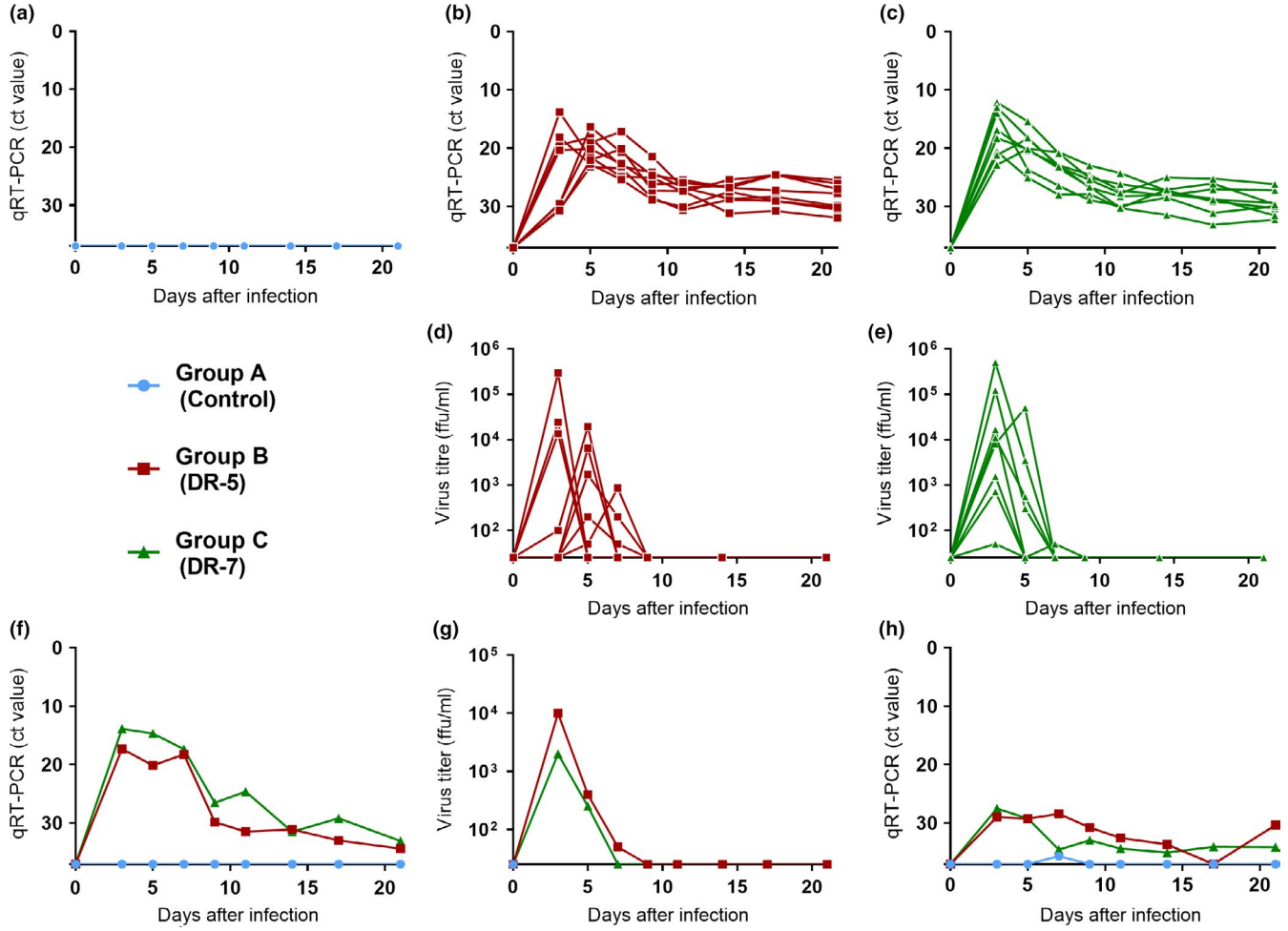

FIGURE 2 Shedding of viral RNA and infectious virus by RVA-inoculated pigeons. (a–c) Viral RNA detected by RT-qPCR in cloacal swabs collected from individual birds. (d,e) Infectious virus titres in cloacal swabs collected from individual birds. (f) Viral RNA and (g) infectious virus detected in pooled faecal samples from each group. (h) Viral RNA detected in drinking water collected from the experimental rooms. The lower limit of the *Y*-axis represents the detection limit of the respective assay

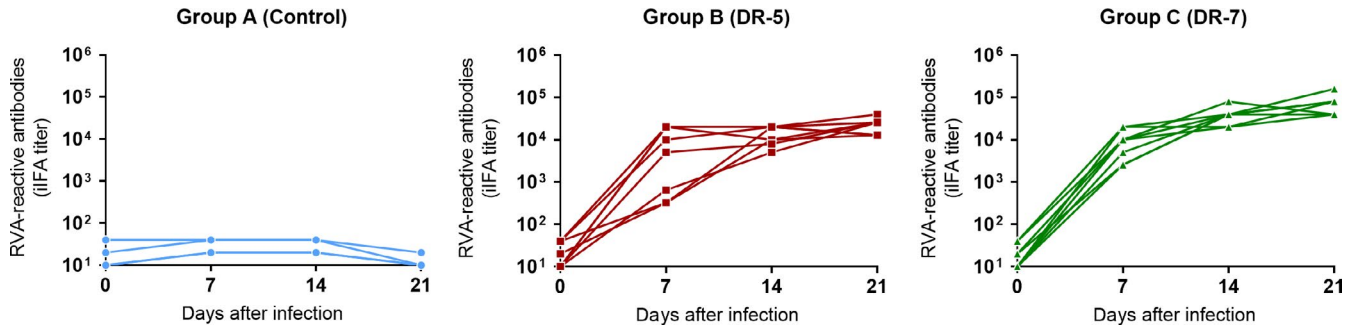

FIGURE 3 Seroconversion of RVA-inoculated pigeons. RVA-reactive antibodies determined by indirect immunofluorescence assay (iIFA) in serum samples collected from individual birds. The lower limit of the Y-axis represents the detection limit of the assay

Low to moderate levels of viral RNA were still detectable in all analysed tissue types with no apparent differences between the two infected groups. Lowest Cq values were detectable in the spleen, whereas detection in blood collected at day 21 p.i. barely exceeded the detection limit of the assay (Figure 4e). In line with the low levels of viral RNA, infectious virus was not detectable in samples collected from liver and intestine at day 21 p.i. (data not shown). No viral RNA was detected in any tissue sample collected from the uninfected group A (Figure 4e).

Liver and bursa collected at necropsy were additionally tested for CoHV-1 or PiCV-1 DNA, respectively. While PiCV-1 DNA was not detectable in bursal tissue, the liver of one animal of group B contained CoHV-1 DNA (data not shown).

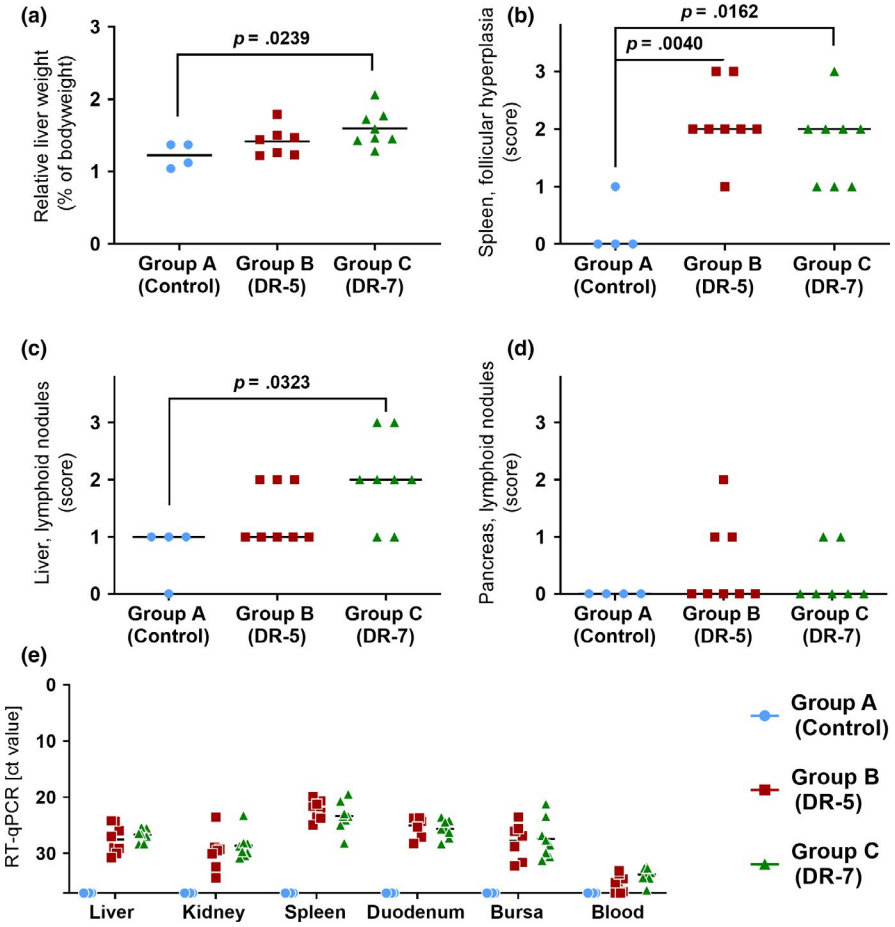

FIGURE 4 Gross and microscopic lesions and detection of viral RNA in tissue samples at day 21 after RVA inoculation. (a) Relative liver weight as percentage of bodyweight. (b–d) Histopathology score representing splenic follicular hyperplasia (b) or lymphoid nodules in liver (c) and pancreas (d). (e) Viral RNA detected by RT-qPCR. The lower limit of the *Y*-axis represents the detection limit of the assay. Black horizontal bars represent the arithmetic mean (a, e) or median (b-d) of the respective group. *P* values below .05 indicate significant differences, as determined by unpaired Student's *t* test (a) or Mann–Whitney test (b–d)

4 | DISCUSSION

RVA genotype G18P[17] variants have been found to be widespread and epidemiologically closely associated with YPDS-like outbreaks in domestic pigeons in Europe and Australia, but formal proof for their causative role was still missing (McCowan et al., 2018; Rubbenstroth et al., 2019). Here we fulfil Henle-Koch's postulates by reproducing YPDS-like disease in experimentally RVA-inoculated juvenile homing pigeons.

In both inoculated groups, an acute, self-limiting disease with 100% morbidity was observed. Clinical signs included apathy, regurgitation, congested crops, diarrhoea, anorexia and weight loss, representing the typical presentation of natural RVA infections in pigeons and YPDS (Gough et al., 1992; McCowan et al., 2018; Rubbenstroth et al., 2019). Surprisingly, clinical signs started already two to three days after inoculation. In this proof-of-principle experiment, we applied a high infectious dose directly into the crop to achieve a uniform infection of all birds. Whether the incubation period observed under these experimental conditions also reflects that after natural RVA infection remains to be elucidated. Acute clinical signs subsided by day 10 p.i., which is in good agreement with field observations. However, full clinical recovery with regained weight took more than 14 days for some birds of the DR-7-inoculated group C. In line with this observation, birds of both infected groups still exhibited splenomegaly and slightly increased liver weights along with an activation of the adaptive lymphatic system, particularly within spleen and liver, at the end of the experiment. The histopathological findings are in agreement with (but do not prove) a previous hepatocellular degeneration and necrosis, as observed for acute natural RVA infections (McCowan et al., 2018; Rubbenstroth et al., 2019). Further studies are required to analyse the course of tissue lesions in more detail.

The long-lasting lesions are of particular relevance from the perspective of animal welfare, since an optimal health condition is mandatory for all birds participating in homing pigeon races infections and RVA infections often occur at the beginning of the racing season of juvenile pigeons (Rubbenstroth et al., 2019). Our results indicate that regaining this condition may take more than 3 weeks after RVA-induced disease, despite supposedly earlier clinical recovery.

Mortality was not observed during this study, whereas the proportion of deceased and euthanized birds may range from none to more than 50% following natural infections (Hunnam et al., 2019; McCowan et al., 2018; Rubbenstroth et al., 2019). Infectious or non-infectious factors contributing to the severity of RVA-induced disease in pigeons are not well characterized. Various infectious agents, including PiCV-1, CoHV-1, and different avian adenoviruses, have been discussed as potential co-factors of YPDS in the past, but clear association has not been demonstrated (Ballmann & Harrach, 2016; Freick et al., 2008; Raue et al., 2005; Schmidt et al., 2008; Stenzel et al., 2012; Teske et al., 2017). In our experimental

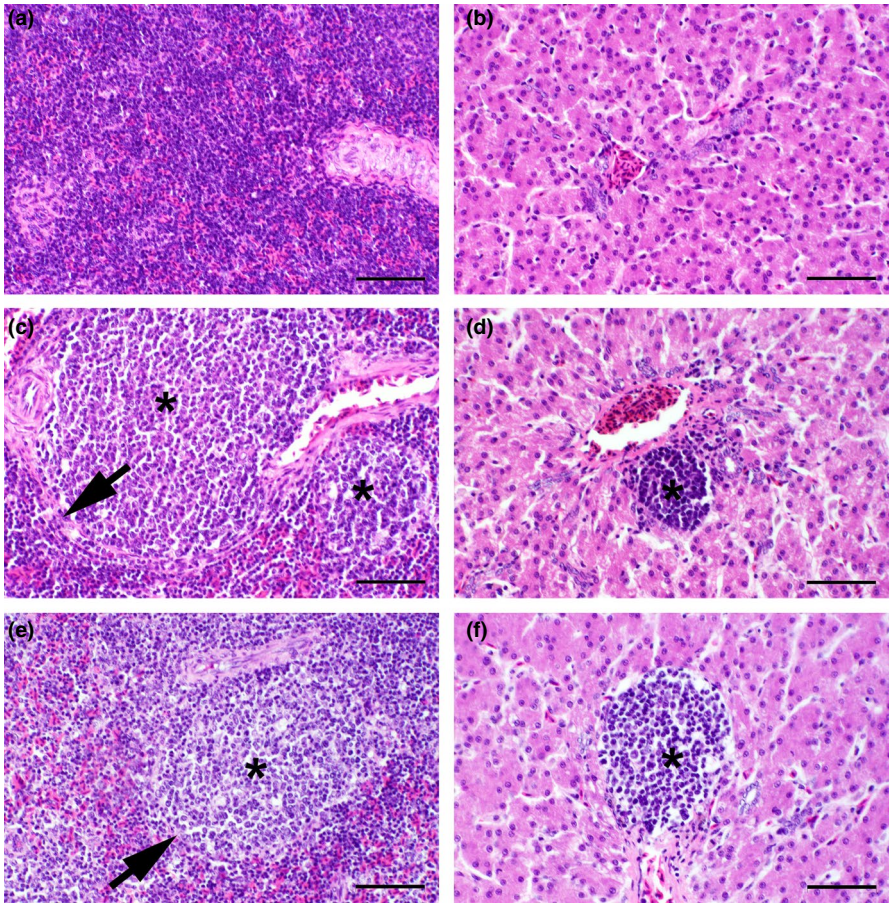

FIGURE 5 Histopathologic findings in livers and spleens at 21 days post-RVA inoculation. Haematoxylin–eosin staining of spleen (panels a, c, e) and liver (panels b, d, f) sections of uninfected control pigeons (group A; panels a, b) compared to pigeons inoculated with RVA isolates DR-5 (group B; panels c, d) or DR-7 (group C; panels e, f), respectively. Spleens of DR-5- and DR-7-infected pigeons showed severe (C) or moderate (e) follicular hyperplasia, respectively, with prominent germinal centres (asterisks) surrounded by a thin connective tissue capsule (arrows), respectively. Livers of DR-5- and DR-7-infected pigeons exhibited moderate (d) or severe (f), ectopic lymphoid nodule formation (indicated by asterisks), respectively, within the periportal regions. Bars = 50 μm

birds, neither PiCV-1 nor pigeon adenoviruses were detectable despite the use of broad range PCR assays for the detection these viruses (Duchatel et al., 2009; Freick et al., 2008; Raue et al., 2002; Schrenzel et al., 2005; Teske et al., 2017). CoHV-1 was detectable only in a single liver sample at the end of the experiment.

The possibility of maternally derived antibodies providing partial protection against RVA-associated disease has been discussed previously (Rubbenstroth et al., 2019). We detected very low levels of presumably maternally derived antibodies in some of the birds at the beginning of the experiment, which is in agreement with their origin from a pigeon loft with a history of annual RVA infections. Whether or not their presence may have influenced the outcome of the experiment remains unknown. In field outbreaks, juvenile pigeons have been described to be fully susceptible to the disease at the age of three months (Rubbenstroth et al., 2019).

Based on field observations, strain-dependent genetic differences are assumed to result in variable virulence profiles of pigeon RVA lineages. Over the past two years, RVA-associated outbreaks with high mortality have been reported more frequently in Germany, coinciding with the emergence of new RVA variants in Europe in 2017 (Rubbenstroth et al., 2019). Isolate DR-7 (lineage 2017a) represents one of those variants and was isolated from a severe outbreak with 12.5% mortality, whereas DR-5 (lineage 2015a) originated from a 'typical' YPDS-like outbreak with mild disease and only 2.5% mortality. In congruence, the DR-7-inoculated group C developed markedly more pronounced clinical signs with a prolonged course of disease as compared to the DR-5-inoculated group B. Genetic virulence determinants of RVA in pigeons are so far unknown. The VP6 and NSP5/6 segments of DR-5 and the VP4 and VP7 segments of DR-7 are phylogenetically closely related to the apparently virulent Australian variant (McCowan et al., 2018; Rubbenstroth et al., 2019), suggesting the latter two gene products as potential virulence factors.

Shedding of viral RNA and infectious virus had peaked already a few days after inoculation, but viral RNA was still detectable in organs, faeces and cloacal swabs at the end of the experiment after three weeks. During field studies, low levels of viral RNA were detectable for up to 12 weeks after confirmed RVA outbreaks (McCowan et al., 2018; Rubbenstroth et al., 2019), but shedding of infectious virus was not determined. In this study, infectious virus in cloacal swabs and pooled faecal samples remained detectable for no longer than seven days after infection and it was likewise absent from liver and intestinal samples collected at day 21 p.i., despite the presence of viral RNA. However, it cannot be excluded that the virus isolation procedure employed in this study does not detect pigeon rotaviruses with sufficient efficiency. Thus, further research is required to determine the duration of potential virus transmission by reconvalescent birds.

In summary, we formally confirm pigeon-associated RVA G18P[17] to be a primary pathogen of juvenile domestic pigeons causing an acute disease compatible with the presentation of YPDS. We further provide an infection model to be used for answering

future questions, including identification of transmission routes and establishing vaccination regimes.

ACKNOWLEDGEMENTS

The authors like to thank Hilke Bartels, Gabriele Czerwinski, Anne Günther, Alicia Linares and Kathrin Steffen for excellent technical assistance, Leonie Forth for conducting the RIEMS analyses and Stefanie Marzahl, Thomas Möritz, Lukas Steinke and Maxi Zander for cautiously taking care of the experimental birds. Part of this work was financially supported by European Union Horizon 2020 Research and Innovation Program COMPARE (Grant Number 643476).

ETHICS STATEMENT

The in vivo experiment was conducted in compliance with the German animal protection law (TierSchG) and approved by the local animal welfare authority (LALLF Mecklenburg-Vorpommern; application number 7,221.3–2-010/18). Housing and handling were performed in accordance with good animal practice as defined by FELASA (http://www.felasa.eu) and the national animal welfare body GV-SOLAS (http://www.gv-solas.de).

CONFLICT OF INTEREST

The authors declare no financial or personal relationships with other people or organizations that could inappropriately influence their work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Rubbenstroth D, Ulrich R, Wylezich C, Rautenschlein S, Beer M, Mohr L. First experimental proof of *Rotavirus A* (RVA) genotype G18P[17] inducing the clinical presentation of 'young pigeon disease syndrome' (YPDS) in domestic pigeons (*Columba livia*). *Transbound Emerg Dis*. 2020;67:1507–1516. https://doi.org/10.1111/tbed.13485