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ORIGINAL ARTICLE

A novel *Chaphamaparvovirus* is the etiological agent of hepatitis outbreaks in pheasants (*Phasianus colchicus)* characterized by high mortality

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Abstract

In the present study, we report the occurrence of several outbreaks of hepatitis in flocks of young pheasants in France, between 2017 and 2021. The disease was characterized by prostration, apathy and a median cumulative mortality of 12%, with the birds presenting multifocal to coalescing necrotizing hepatitis on necropsy. Severe extensive areas of degeneration and necrosis were observed in the liver, with degenerative hepatocytes presenting large amphophilic to acidophilic intranuclear inclusion bodies. Transmission electron microscopy examination of liver samples showed the presence of parvovirus-like virions of 21–24 nm, a finding already reported decades ago. Further investigations by Next Generation Sequencing and PCR revealed the complete genome of a novel species of parvovirus, here designated *Phasianus chaphamaparvovirus 1* (PhChPV-1), that belongs to the new genus *Chaphamaparvovirus* in the *Hamaparvovirinae* subfamily. In situ hybridization and real-time PCR confirmed the etiology of the outbreaks, demonstrating the viral genome in the lesions. The findings establish the etiology of a pathology first described in pheasants 50 years ago and pave the way for a targeted protection strategy.

KEYWORDS
Chaphamaparvovirus, hepatitis, inclusion bodies, outbreaks, pheasants

1 | INTRODUCTION

The common pheasant (*Phasianus colchicus*) is an ornamental game bird present in different parts of the world that belongs to the class of Aves, order Galliformes, family *Phasianidae*, genus *Phasianus* (Robertson & Hill, 1988). Pheasants can be subdivided in 30 subspecies, according to plumage and geographical area (Madge et al., 2002). As game birds, pheasants are usually reared semi-intensively or extensively in farms, being later released into the wild to be hunted, which is an activity that has increased in the last decades (Horigan et al., 2014). Such husbandry systems present more biosecurity challenges than conventional poultry farms, implying a higher risk of introduction and transmission of pathogens. In this regard, pheasants are susceptible to various pathogens typically described in chickens and turkeys, who are

also members of the family *Phasianidae*. This includes diseases such as avian influenza, Newcastle disease, infectious bursal disease, infectious laryngotracheitis, avian botulism, fowl cholera, mycoplasmosis, salmonellosis and coccidiosis, as summarized recently by Dwight et al. (2022, appendix S1). Additionally, marble spleen disease (MSD) caused by the *Turkey siadenovirus A* (TAdV-3), a member of the *Siadenovirus* genus, is of great importance in pheasants, producing a characteristic pathology picture, which includes enlarged, mottled (marbled) spleen and the presence of intranuclear inclusions within mononuclear cells (Rautenschlein et al., 2020). Intranuclear inclusions have been additionally described in hepatitis outbreaks in pheasants in the United States and Italy, with one report associating such pathology with the presence of a parvovirus in the hepatocytes by electron microscopy (Gelmetti et al., 1996; Rosen et al., 1965).

Parvoviruses are small, icosahedral, non-enveloped viruses, which have a linear, single-stranded DNA (ssDNA) genome, and belong to the large and diverse *Parvoviridae* family (Cotmore et al., 2019). Poultry, especially young birds, are susceptible to infections from parvoviruses within the *Parvovirinae* subfamily. Thus, chicken and turkey parvoviruses, within the genus *Aveparvovirus*, are responsible for enteric disease, and are often associated with the runting-stunting syndrome and the poult enteritis complex, respectively, although they might be present in healthy birds as well (Day, 2020). Additionally, strains from the *Anseriform dependoparvovirus 1* species, within the genus *Dependoparvovirus*, are responsible for a highly contagious and fatal disease in young geese and Muscovy ducks, and a less severe, chronic disease in mule and Pekin ducks (Palya, 2020). In 1993, the *Parvoviridae* family was divided in two subfamilies, based on host association, with the subfamily *Parvovirinae* consisting of viruses that infect vertebrates, and the subfamily *Densovirinae* comprising viruses that infect invertebrates (Cotmore et al., 2019). However, since 2012, genomes belonging to a novel lineage of parvoviruses designated 'Chapparvovirus' were detected in different vertebrates. For that reason, the family *Parvoviridae* was recently reorganized and a new subfamily – *Hamaparvovirinae* – was created, which accommodates viruses infecting vertebrates and invertebrates (Pénzes et al., 2020).

In the present report, we describe a series of field outbreaks of severe hepatitis in young pheasants from rearing farms in France, caused by a novel *Chaphamaparvovirus*, from the recently created *Hamaparvovirinae* subfamily, culminating in high mortality and severe losses.

2 | MATERIALS AND METHODS

2.1 | Case history and post-mortem investigations

Between 2017 and 2021, similar outbreaks were noted in 15 pheasant rearing farms in France, with a clinical picture that consisted of prostration, apathy and increased mortality. Most of the affected flocks were located in the region Pays de la Loire, with two flocks located in the regions of Bretagne and Nouvelle Aquitaine, and comprised between 1000 and 13,000 pheasants. The onset of clinical signs was observed when the birds were 3–6 weeks old, and the cumulative mortality varied between 4% and 41%, with a median value of 12% (Table 1). Culled birds were necropsied according to a standard protocol in the laboratory facilities of Labovet and gross pathologic lesions were recorded. Samples mostly from liver, but also from spleen, pancreas, intestine and kidney were collected and processed for histopathology, electron microscopy and molecular investigations.

2.2 | Histopathology

Samples of liver, spleen, intestine and kidney were fixed in 4% neutral buffered formaldehyde solution (SAV LP GmbH, Flintsbach, Germany). Following fixation, samples were dehydrated and embedded in paraffin. Then, the paraffin-embedded samples were cut into 4-μm-thick sections in a microtome (Microm HM 360; Microm Laborgerate GmbH, Walldorf, Germany), and the generated tissue sections were mounted on glass slides and stained with haematoxylin and eosin (H&E) for microscopic assessment.

2.3 | Transmission electron microscopy (TEM)

Previously frozen liver samples, at −20°C, were used for transmission electron microscopy (TEM) examination. The samples were cut in 1 mm^3 sections and fixed in 5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (Sigma-Aldrich, Vienna, Austria), pH 7.2, at 4°C for 3 h. Afterwards, samples were post-fixed in 1% osmium tetroxide (Merck) in the same buffer at 4°C for 2 h. After dehydration in an alcohol gradient series and propylene oxide (Merck), the tissue samples were embedded in glycid ether 100 (Serva, Heidelberg, Germany). The ultrathin sections were cut on a Leica Ultramicrotome (Leica Ultracut S, Vienna, Austria) and stained with uranyl acetate (Sigma-Aldrich) and lead citrate (Merck). Ultrathin sections were examined with a Zeiss TEM 900 electron microscope (Carl Zeiss, Oberkochen, Germany) operated at 60 kV.

2.4 | Nucleic acid extraction and construction of a NGS library

Nucleic acids were extracted from formalin-fixed paraffin-embedded (FFPE) liver and spleen samples from cases 18–06174, 19–03914 and 19–03915/2, using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Vienna, Austria), and from fresh samples of liver, spleen and kidney from case 19–10864 using the DNeasy Blood and Tissue Kit (QIAGEN). The sample from case 19–03914 was used for next-generation sequencing (NGS). Since the DNA extracted from paraffin embedded tissue was already fragmented, no fragmentation prior to NGS-library preparation was needed and the sample was subsequently treated with NEBNext® FFPE DNA Repair Mix (New England BioLabs, Hitchin, UK) according to the manufacturer's instructions. The library was prepared by NEBNext® Ultra II DNA Library Prep Kit for Illumina (New England

TABLE 1 List of outbreaks in pheasant rearing farms in France, between 2017 and 2021, and descriptive information of each affected flock

Case no.	Year	Region	No. of birds in the flock	Vaccination scheme		Age at outbreak onset (weeks)	Cumulative mortality (%)	
				Disease	Age in weeks			(Median)
17-13149	2017	Pays de la Loire	3150	ND[†]	1	4	9	(12)
				MSD[‡]	3–4			
				ND	3–4			
17-13150	2017	Pays de la Loire	3024	ND	1	3	8	
				MSD	4–5			
				ND	4–5			
17-14451	2017	Pays de la Loire	4800	MSD	5	3	4	
18-06173	2017	Pays de la Loire	4000	ND	3–4	4	n.d.[§]	
				MSD	3–4			
18-06174	2017	Pays de la Loire	6000	n.v.[¶]	n.a.[††]	4	n.d.	
19-03914	2018	Pays de la Loire	2700	ND	3–4	6	41	
				MSD	3–4			
19-03915/1	2018	Pays de la Loire	2000	MSD	3–4	3	10	
19-03915/2	2018	Pays de la Loire	1000	MSD	3–4	3	15	
19-10864	2019	Pays de la Loire	5000	ND	1	5	12	
				MSD	3–4			
				ND	3–4			
20-18963	2020	Pays de la Loire	13000	ND	3–4	4	11	
				MSD	3–4			
20-18964	2020	Nouvelle Aquitaine	6192	ND	1	4	20	
				MSD	4			
				ND	4			
20-18965	2020	Pays de la Loire	1368	ND	3	4	20	
				MSD	3			
20-18966	2020	Bretagne	7000	n.v.	n. a	3	n.d	
20-18967	2020	Pays de la Loire	7000	ND	3–4	4	38	
				MSD	3–4			
20-18968	2020	Pays de la Loire	2200	ND	3–4	3	33	
				MSD	3–4			
21-13344	2021	Pays de la Loire	3670	ND	1	3	8	
				ND	3			
				MSD	3			

[†]Newcastle disease.
[‡]Marble spleen disease.
[§]Not determined.
[¶]Not vaccinated.
[††]Not applicable.

Biolabs) according to the manufacturer's instructions. The size distribution of the constructed NGS-library, with a narrow peak of approximately 300 bp, was identified with the Agilent High Sensitivity DNA Kit (Agilent Technologies, Vienna, Austria) based on the manufacturer's instruction, using the Agilent Bioanalyzer 2100 (Agilent Technologies). The NGS-library was sequenced using 150-bp paired-end read mode on the NextSeq platform (Illumina) at Vienna BioCenter Core Facilities GmbH (Next Generation Sequencing Facility, Vienna, Austria).

2.5 | Analysis of NGS-data obtained from NextSeq

The de-multiplexed raw NGS-data were analysed with an in-house established bioinformatics pipeline. In short, the NGS-reads were adaptor trimmed using cutadapt (Marcel Martin, 2011) and assembled with MEGAHIT (default parameters). The resulting metagenomics contigs were divided into 200-bp non-overlapping windows and analysed by the Taxonomic Profiling tool from CLC Genomics Workbench 20

(QIAGEN), against a precompiled database containing all viral and bacterial sequences from NCBI (April 2020). Contigs having at least one window mapping to viruses were extracted and BLASTed ($p < 10^{-3}$) to the NCBI complete viral genomes database downloaded in April 2020, which contained 12,156 viral sequences. Read depth for each contig was calculated by aligning the total reads of each sample to the contigs with Bowtie2 (default parameters). To validate the correctness of the BLAST hits, contigs of each viral species were manually BLASTed to the nucleotide database and hits confirming the automatic classification were retained. To identify species of clinical relevance, phages and chicken endogenous viruses were filtered out from the final summary of viral species present in each sample. All metagenomic contigs confirmed as avian parvovirus sequences were mapped to the representative strain of the Muscovy duck parvovirus genome (strain FM – NCBI accession NC_006147).

2.6 | Creating the complete genome sequence

Since the initial assembly with NGS-derived reads produced only a partial genome sequence (1554 bp), the remaining sequence, including gaps and genome ends, was amplified by PCR, cloned into the PCR-Vector and sequenced with the Sanger method. In order to have sufficient DNA for the analyses, liver tissue from sample 20–18963, which was positive for parvovirus by real-time PCR, was used instead of the FFPE sample from case 19–03914. Prior to DNA extraction, the sample was pre-treated (Technical Appendix – Section 1) and DNA was extracted with the DNeasy Blood and Tissue Kit (QIAGEN), according to the manufacturer's instructions. Six PCRs were used to close the gaps whereas specific nested PCRs were applied to amplify the 5′- and 3′-ends of the genome (Technical Appendix – Section 1). Finally, the nearly complete genome was amplified with primers PhChPV-1-compl-F and PhChPV-1-compl-R (Technical Appendix – Section 1) and high fidelity DNA polymerase.

Expected PCR products (Appendix Table A1) were excised and purified from gel with QIAquick Gel Extraction Kit (QIAGEN), according to the manufacturer's instructions. Purified fragments were cloned into the into pCR-Blunt II-TOPO vector by using the Zero Blunt TOPO Cloning Kit for sequencing (Invitrogen, Thermo Fisher Scientific, Vienna, Austria) according to the manufacturer's instructions. Three positive clones per sample were sequenced by Sanger sequencing using M13 primers (LGC Genomics, Berlin Germany). Assembly and analyses of sequences were performed with Acccelrys Gene, version 2.5 (Accelrys, San Diego, CA) software. Complete genome sequence was submitted to NCBI database under the accession number MZ667220.

2.7 | Genome analysis

The secondary structure of 5′- and 3′-UTR regions of the genome was predicted using the Quikfold application on the UNAfold Web Server (http://www.unafold.org/Dinamelt/applications/quickfold.php) with default parameters. The open reading frames (ORFs) were predicted with the fgenesV0 application for Gene Finding in Viral genomes within the Softberry software (http://www.softberry.com/berry.phtml?topic = virus0&group = programs&subgroup = gfindv) using generic parameters for Markov chain-based gene prediction, linear genome and standard genetic code.

2.8 | Phylogenetic analysis

The phylogenetic analysis was based on the complete NS1 protein sequences (deduced amino acids). For that purpose, 51 NS1 protein sequences including at least one species representative of all genera belonging to subfamilies of *Hamaparvovirinae* and *Parvovirinae* were downloaded from the NCBI protein database. The amino acid alignment was produced by using the MUSCLE program implemented in the MEGA X software with Neighbour Joining Clustering method (Kumar et al., 2018). The phylogenetic analysis was inferred with maximum-likelihood method and Le and Gascuel model (Le & Gascuel, 2008) using MEGA X software. The robustness of the phylogenetic tree is based on bootstrap values generated from 500 replicates. Sequence distances of NS1 proteins within *Hamaparvovirinae* subfamily members were determined by MegAlign module of Lasergene v12 software using ClustalW alignment (DNASTAR, Madison, Wisconsin, USA) applying default slow/accurate alignment parameters and Gonnet Series protein weight matrix.

2.9 | Real-time PCR and in situ hybridization

In order to examine the samples for PhChPV-1, DNA was extracted from fresh organs and FFPE samples with the DNeasy Blood and Tissue Kit (QIAGEN) and the QIAamp DNA FFPE Tissue Kit (QIAGEN), respectively, according to the manufacturer's instructions. Primers and probe targeting a NS1 protein sequence (Table 2) were used in a real-time PCR to detect PhChPV-1 DNA in the investigated samples. The real-time PCR was performed in an Agilent AriaMx (Agilent Technologies) using a Brilliant III Ultra-Fast QPCR Master Mix with Low ROX kit (Agilent Technologies). Each 20 μl reaction mixture consisted of 2 μl template DNA, a final concentration of 300 nmol/L of each primer (forward and reverse) and a final concentration of 250 nmol/L of probe. The real-time PCR cyclic conditions consisted of an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 s and combined annealing/extension step at 60°C for 10 s, with the fluorescence data being collected during the latter step. Data were analysed in the Agilent AriaMx software version 1.7 (Agilent Technologies) by setting the threshold automatically. The absence of contamination in the assay was monitored by the inclusion of a no template control.

Paraffin-embedded samples were investigated by in situ hybridization (ISH), using a digoxigenin-labelled specific DNA probe based on the NS1 gene of PhChPV-1 (Table 2), and following a protocol earlier described Liebhart et al. (2006).

TABLE 2 List of primers and probes used for real-time PCR and ISH investigations

Targeted gene	Primer/probe	Sequence (5′ → 3′)	Method
PhChPV-1 NS1	Forward primer	GAA CGC TGG TTC CTG ATC CTT C	Real-time PCR
	Reverse primer	CCC GTG TTT GAC GGT CCA TAG	
	Probe	TGC ATT CTC TTG CAT CAA GGA GTT AAC CCT TCT G	
	Probe	CCC GTG TTT GAC GGT CCA TAG ATG TGG ATG CAG TTG CG	ISH

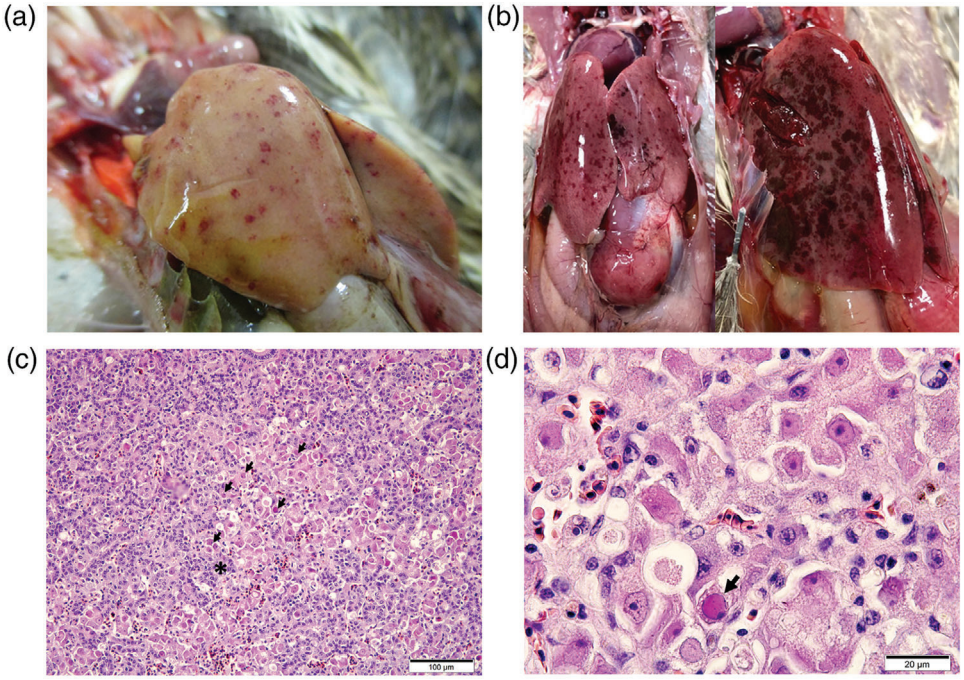

FIGURE 1 Post-mortem and histopathology investigations. Diseased birds presented severe hepatitis that varied in appearance: (a) swollen, mottled, yellowish-brown livers and (b) haemorrhagic hepatitis with multiple ecchymotic lesions on the liver surface. The histopathology investigation revealed (c) extensive areas of necrosis in liver, with infiltration of mononuclear inflammatory cells and heterophils (*) and degenerated hepatocytes presenting eosinophilic intranuclear inclusion bodies (→) (H & E; bar = 100 μm). A high magnification shows (d) an eosinophilic intranuclear inclusion body in detail (→), surrounded by degenerating hepatocytes and inflammatory cells (H & E; bar = 20 μm)

3 | RESULTS

3.1 | Post-mortem and histopathology findings

At necropsy, birds from impaired flocks revealed hepatitis characterized by hepatomegaly, with changes in the organ appearance varying from mottled to yellowish-brown, with 20–30% of birds also presenting multifocal to coalescing depressed red foci in the parenchyma (Figure 1a and b). Approximately 80% of birds presented mild to severe haemorrhagic enteritis, nephritis, with 10–20% of birds presenting congested and swollen spleens as well. The histologic investigation showed multifocal to almost diffuse degeneration and necrosis of hepatocytes (most frequently as random hepatocyte necrosis), with generally sparse infiltration by mononuclear inflammatory cells and heterophils, and with degenerating hepatocytes presenting amphophilic to acidophilic prominent intranuclear inclusion bodies, filling the nucleoplasm (Figure 1c and d). In subacute to chronic forms, which were regularly observed, a moderate to marked hyperplasia of bile ducts was also noticed. In some birds, an inflammation of the intestinal mucosa by mononuclear cells could be observed.

3.2 | Discovery of a novel *Chaphamaparvovirus* in pheasants by TEM and NGS

The TEM examination of liver samples revealed the presence of small, icosahedral viral particles, approximately 21–24 nm in diameter (Figure 2). Morphology and regular particle size suggested their identification as parvovirus virions.

The metagenomic profiling using de novo assembled contigs of the FFPE sample from case 19–03914 identified different families of bacteria and viruses (Figure 3a). Since the clinical picture suspected a viral

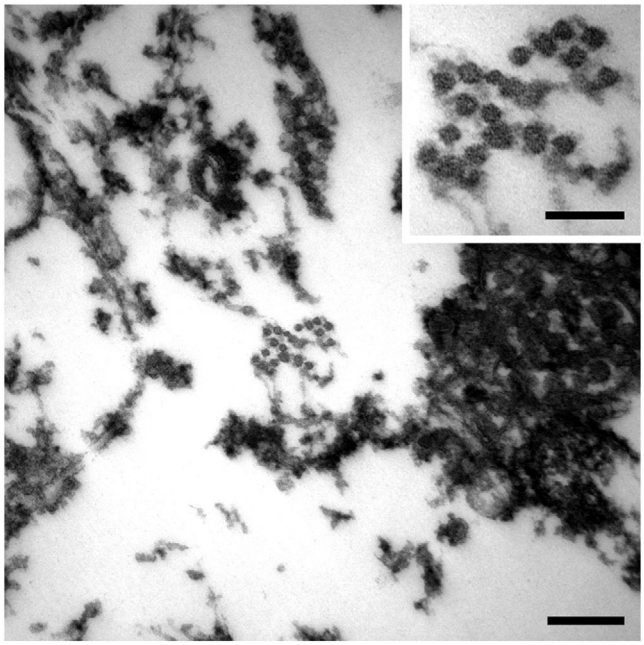

FIGURE 2 TEM micrograph of a liver sample revealing small, icosahedral viral particles of 21–24 nm. The insert represents a higher magnification of the particles. Scale bars = 250 nm and 100 nm (insert)

etiology, a detailed characterization of viral contigs was performed by computing the genomic coverage of viral species of clinical interest (Appendix Table A2). Even though the majority of viral contigs mapped to dsDNA viruses, more precisely to TAdV-3 (haemorrhagic enteritis virus [HEV]/marble spleen disease virus [(MSDV]), this finding was discarded as potential etiological agent since birds received the live vaccine against this virus. Considering the etiology and the observed lesions, in particular inclusion bodies in hepatocytes and parvovirus-like virions, the focus was made on the detection of the parvovirus. For that purpose, viral contigs were BLASTed against an in-house generated database consisting of all known parvovirus sequences. Contigs confirmed as avian parvovirus sequences were mapped to the Muscovy duck parvovirus genome, which produced a preliminary low-coverage assembly (Figure 3b).

3.3 | Genome structure analysis

In order to complete the genomic sequence, a set of PCRs was developed (Appendix Table A1), which resulted in the final whole genome sequence of 4546 bp. The genome consists of 5′- and 3′-untranslated regions (UTRs) and 5 open reading frames (ORFs) (Figure 4a). The Quikfold analysis (http://www.unafold.org/Dinamelt/applications/quickfold.php) of both 5′- and 3′-ends suggested hairpin secondary structures at both ends (Figure 4b), indicating that the obtained genomic sequence appears to be complete. Four partially overlapping ORFs are located in the first half of the genome after the 5′-UTR, with the longest ORF showing the highest homology to NS1 proteins of parvoviruses (Figure 4a). The second half of the genome contains a single ORF which is orthologous to VP1 proteins of parvoviruses. The predicted NS1 protein is 673 amino acids long and contains a SF3 helicase1 domain (PS51206) (amino acid 299–443) found in NS1 proteins of parvoviruses. The VP1 sequence is 562 amino acids in length and, according to InterProScan and ScanProsite, contains no specific protein domains. The genome structure of this parvovirus seems overall similar to the other reported *Chaphamaparvovirus* genomes (Liu et al., 2020). However, unlike *Chaphamaparvoviruses* recently identified in North American wild ducks, no coding sequence for VP2 could be identified (Canuti et al., 2021).

3.4 | Phylogenetic analysis

Phylogenetic analysis based on the NS1 protein revealed that the genome identified in pheasants clusters to the *Chaphamaparvovirus* genus of the newly recognized subfamily *Hamaparvovirinae* (Figure 5). According to the recently proposed taxonomy, it appears to be a representative of a separate species, here designated *Phasianus chaphamaparvovirus 1* (PhChPV-1), as the closest NS1 sequence in the database (Parvovirus partridge/PA147/ITA2008 [ADZ48579]) shows less than 85% amino acid sequence identity (82.1%; Appendix Table A3) (Pénzes et al., 2020).

3.5 | Detection of *Phasianus chaphamaparvovirus* genome in tissues by real-time PCR and ISH

FFPE organ samples from cases 18–06174, 19–03914 and 19–03915/2, together with unfixed organ samples from cases 19–10864, 20-18963-8 and 21–13344, were investigated by real-time PCR with a fluorescent probe targeting the NS1 gene of PhChPV-1 (Table A3). All investigated samples became positive for PhChPV-1 DNA, with threshold cycles (C_ts) varying from 16.28 to 38.48. Tissue samples from cases 18–06174, 19–03914, 19–03915/2 and 20-18963-18966 were further investigated by ISH using a probe targeting the NS1 gene of PhChPV-1. Strong and clear signals were observed in sections of liver from all investigated samples, particularly in the intranuclear inclusion bodies (Figure 6). No positive signals were noted in other investigated organs.

4 | DISCUSSION

In the present study, we report the occurrence of several outbreaks of hepatitis in flocks of young pheasants in France, between 2017 and 2021, caused by a novel species of parvovirus, within the recently created subfamily *Hamaparvovirinae*. The outbreaks were characterized by increased and substantial mortality, prostration and apathy in the afflicted flocks, with the birds presenting multifocal to coalescing necrotizing hepatitis during necropsy. One of the most remarkable features at microscopic examination was the presence of intranuclear inclusion bodies in the liver of the diseased birds. Inclusion body

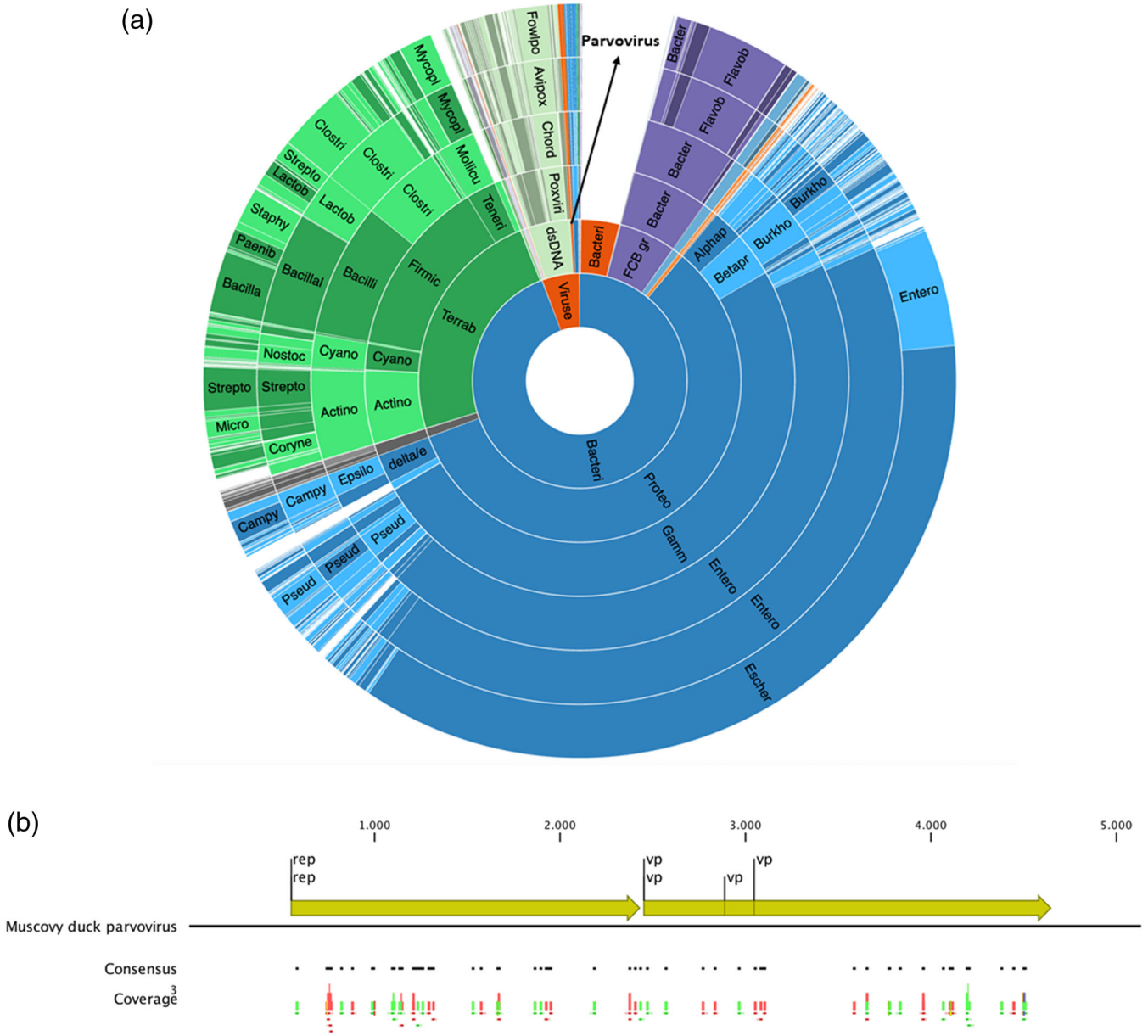

FIGURE 3 (a) Metagenomic profiling of sample from case 19–03914 showing the presence of multiple viral and bacterial species. The arrow points to the tiny fraction of the viral content that was classified as parvovirus. (b) Alignment of 200-bp non-overlapping windows derived from metagenomic contigs to the representative strain of the Muscovy duck parvovirus (strain FM) highlighting the low coverage of the preliminary assembly derived from NGS data

hepatitis (IBH) is a very well documented and studied disease in poultry, specifically in young chickens, which is caused by fowl adenoviruses (FAdVs) (Hess, 2020). FAdVs, which are known to infect various bird species (Fitzgerald, 2020), were once isolated in young pheasants but from the respiratory tract (Cąkała, 1966). In the present report, however, the etiologic role of FAdVs in the hepatitis outbreaks was ruled out by cell culture and PCR investigations on different organ tissues (Technical Appendix – Section 2). Alternatively, parvovirus strains from the *Anseriform dependoparvovirus 1* species, within the *Dependoparvovirus* genus, are capable of causing acute disease in young geese and ducks, with characteristic lesions including hepatitis and the presence of similar intranuclear inclusion bodies (Palya, 2020). Interestingly, early reports described outbreaks of highly infectious viral hepatitis in young pheasants, with an analogous clinical presentation to the present study, which included high mortality, hepatomegaly with congestion and inclusion bodies in the hepatocytes (Rosen et al., 1965; Swarbrick, 1973). However, the etiology of the problem was not determined at the time. Similar to our investigation, Gelmetti et al. (1996) observed parvovirus-like particles by negative staining electron microscopy (EM) in the liver of young pheasants from three rearing facilities suffering also from high mortality and presenting haemorrhagic hepatitis with inclusion bodies. Hence, the present

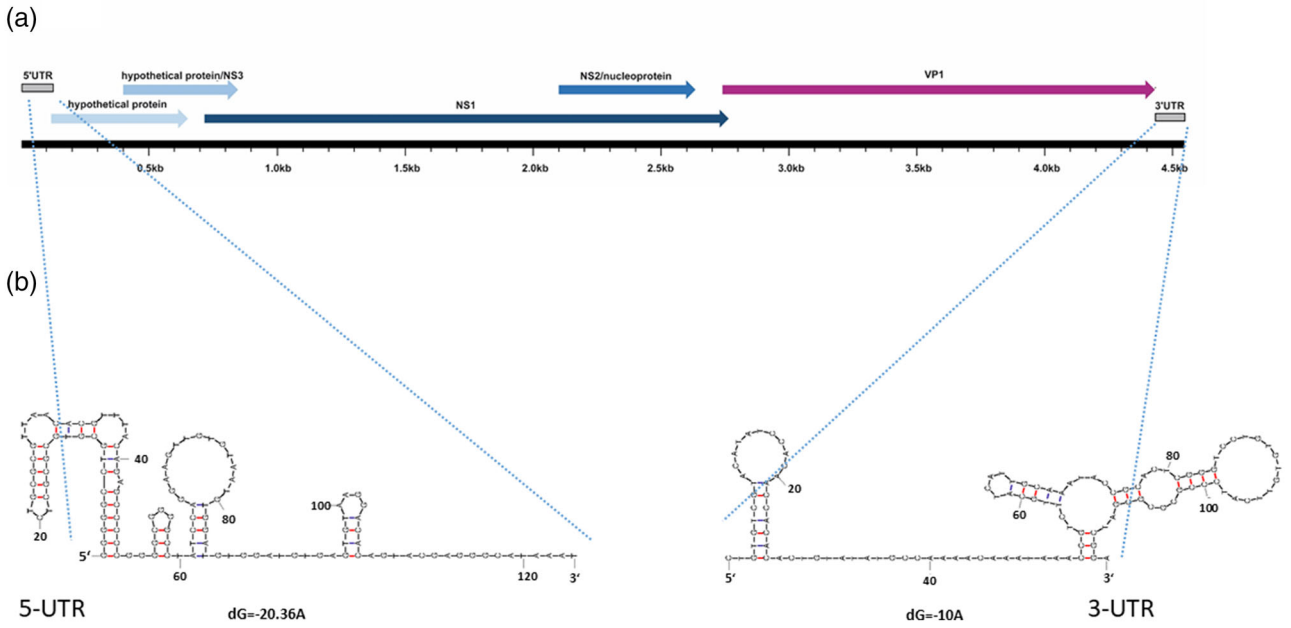

FIGURE 4 PhChPV-1 genome characteristics. (a) Schematic representation of PhChPV-1 genomic features with in silico predicted ORFs. (b) The predicted secondary structure of the terminal repeats of PhChPV-1

TABLE 3 Description and results of investigated samples by ISH and real-time PCR for PhChPV-1

			Real-time PCR	
Case no.	Investigated sample	ISH	Result	C_t[†] value
18-06174	FFPE[‡] organs (liver, intestine, spleen and pancreas)	n.d.[§]	+[¶]	20.97
19-03914	FFPE organs (liver and bursa of Fabricius)	+	+	17.31
19-03915/2	FFPE organs (liver and spleen)	+	+	19.87
19-10864	Liver	n.d.	+	25.96
	Spleen	n.d.	+	30.95
	Kidney	n.d.	+	29.21
20-18963	Liver	+	+	16.28
20-18964	Liver	+	+	19.73
20-18965	Liver	+	+	20.63
20-18966	Liver	+	+	19.34
20-18967	Liver	n.d.	+	17.97
20-18968	Liver	n.d.	+	38.48
21-13344	Liver	n.d.	+	18.29

[†]Threshold cycle.
[‡]Formalin-fixed paraffin-embedded.
[§]Not done.
[¶]Positive.

investigation provides a conclusive step in establishing parvovirus as the likely etiology agent of acute haemorrhagic hepatitis with inclusion bodies in pheasants reported from several countries.

Advances in molecular diagnostic tools, such as NGS, enabled the discovery of new viruses, and in particular new parvoviruses, with some of them being identified in avian species (Kim et al., 2020; Liu et al., 2020; Pénzes et al., 2020; Vibin et al., 2020; Wang et al., 2019). Most of these new parvoviruses were classified within the recently established *Chaphamaparvovirus* genus, within the newly created *Hamaparvovirinae* subfamily, which accommodates parvoviruses that

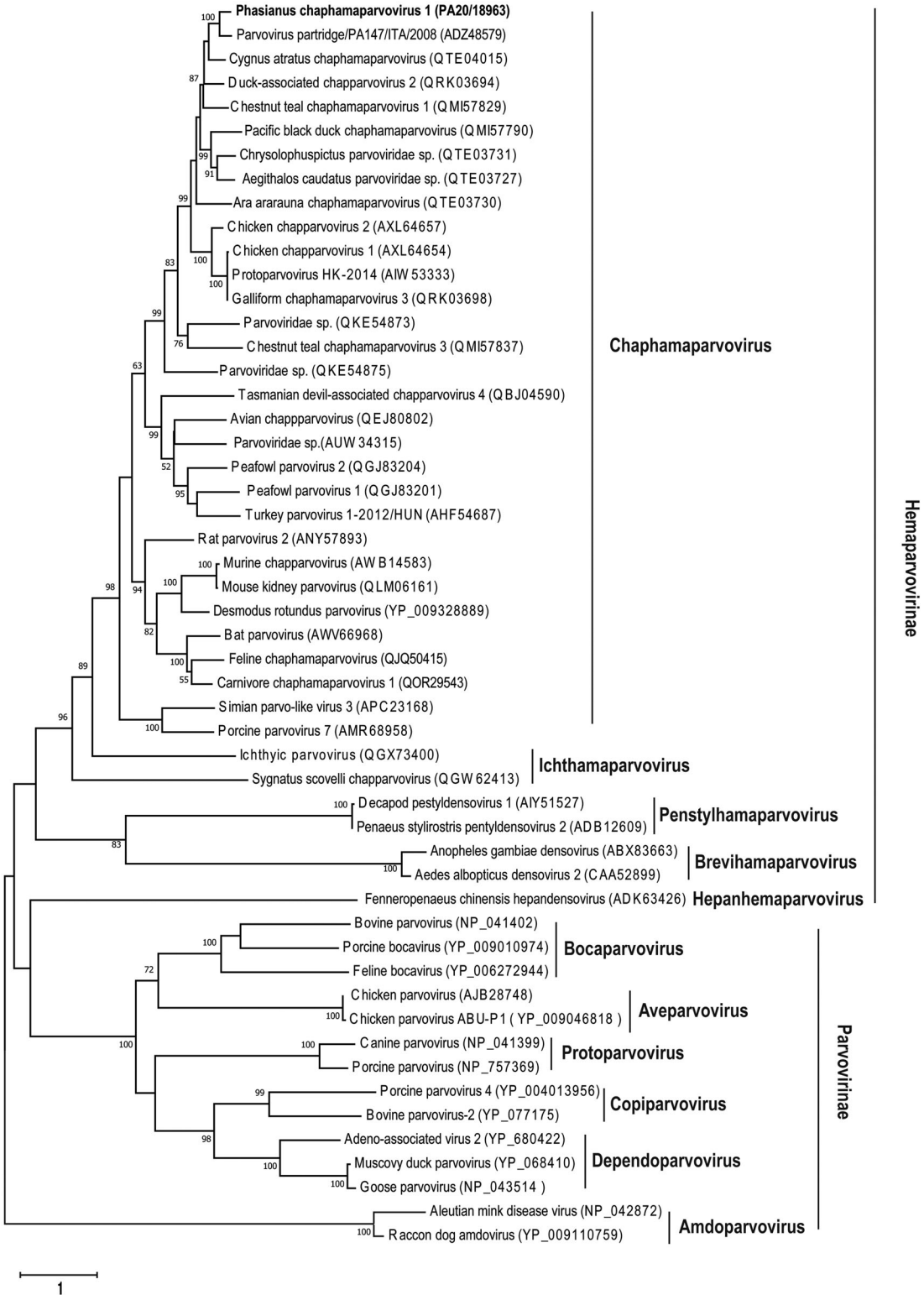

FIGURE 5 Phylogenetic analysis. The evolutionary history was inferred by using the maximum-likelihood method and Le_Gascuel_2008 model (Le & Gascuel, 2008). The tree with the highest log likelihood (−61123.54) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 1.6388]). The rate variation model allowed some sites to be evolutionarily invariable ([+I], 0.31% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 52 amino acid sequences. Accession numbers for NS1 are given in parenthesis next to each sequence except the newly derived PhChPV-1. There were 1242 positions in total in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018)

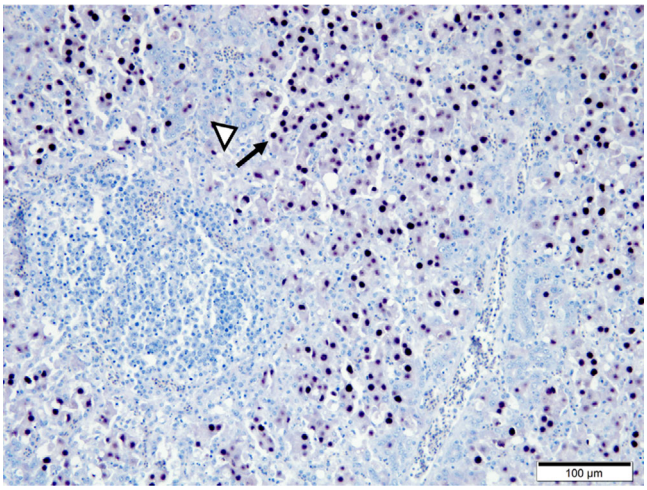

FIGURE 6 Demonstration of PhChPV-1 DNA in liver by ISH. Generalized and strong positive signals (arrow) in histologic section of a liver of a dead pheasant from case 20–18963. The arrowhead labels a non-stained nucleus (ISH bar = 100 μm)

infect both vertebrate and invertebrate hosts, and have an amino acid sequence identity for the NS1 protein above 30% (Pénzes et al., 2020). Likewise, in the present study a new parvovirus – PhChPV-1 – belonging to the *Chaphamaparvovirus* genus was identified by NGS in liver samples from dead pheasants. According to the updated taxon demarcation criteria, parvoviruses can be considered members of different species if their NS1 proteins share less than 85% amino acid sequence identity (Pénzes et al., 2020), thus the PhChPV-1 identified in the present report was categorized in a newly suggested species – *Phasianus chaphamaparvovirus* 1. Interestingly, the closest phylogenetic sequence to PhChPV-1, with 82.1% amino acid sequence identity, is a partial genome sequence of parvovirus associated with hepatitis in grey partridges, in which eosinophilic inclusion bodies were observed in the liver, thus similar to our observations in pheasants (Grilli et al., 2008). Although partridges were initially categorized in a distinct subfamily from pheasants, more recent molecular phylogeny data revealed that these two bird subfamilies actually constitute only one lineage, with some partridges being more closely affiliated with pheasants (Kimball et al., 1999). Therefore, considering that the outbreak in the partridge-rearing farm was observed in the same geographic region as identical parvovirus hepatitis outbreaks in pheasants reported by Gelmetti et al. (1996), it can be hypothesized that such parvoviruses can cross different host species. Interestingly, early on, chukar partridges were implied in the introduction of the pathogen responsible for outbreaks of highly infectious hepatitis in young pheasant farms (Rosen et al., 1965), which aligns with our hypothesis. Although in our study, parvovirus outbreaks were not observed in nearby farms of grey and red partridges, future epidemiologic studies shall include this species as well, as they might act as asymptomatic carriers.

The clinical significance of most newly discovered *Chaphamaparvovirus* identified by NGS in avian species remains unknown, as causation between the virus and a disease was not fully established. Thus, in addition to NGS investigations, in the present study we assessed organ tissues by ISH and real-time PCR, using specific primers and probes targeting the PhChPV-1 NS1 gene. As a result, all investigated samples were PCR positive and hepatocytes with intranuclear inclusion bodies became strongly positive by ISH, indicating a direct causation between PhChPV-1 and the observed pathology. Diseased birds also presented mild to severe haemorrhagic enteritis during post-mortem investigations, which might be explained by the fact that parvoviruses favour infection of actively dividing cell populations such as the enterocytes in young birds (Cotmore et al., 2019). However, no intranuclear inclusion bodies or clear positive signals by ISH were observed in intestinal tissues or in any other investigated organ. Alternatively, Liu et al. (2020) applied immunohistochemistry to investigate the presence of the newly identified peafowl parvovirus 1 in a dead peafowl, by targeting the viral structural protein 1 with mouse polyclonal antibody, revealing positive signals among different tissues, mostly in inflammatory cells. Hence, the demonstration of the pathogen in association with the microscopic lesions, highlight the importance of further studies on *Chaphamaparvoviruses*, as this genus seems to harbour important pathogenic viruses for a diverse range of avian species.

The affected farms from this study produce pheasants for landscape repopulation that are released into the wild from 20 weeks of age onwards. Even though the disease seems to affect young birds up to 6 weeks of age, the etiologic resolution of the outbreaks here reported stresses the need for further epidemiologic studies. Furthermore, new outbreaks are reported every year during the rearing period, which might be due to the high environmental resistance of parvoviruses (Cotmore et al., 2019). For this reason, new intervention strategies are needed regarding farm biosecurity and hygiene. However, game bird farms present numerous structural challenges hindering the application of such measures, as husbandry and management is not comparable with modern poultry farms. Alternatively, a vaccination strategy might be developed, which implies the culture and isolation of the virus in the laboratory.

In conclusion, in the present study, we report the occurrence of several outbreaks of hepatitis in young pheasants in France, which resulted in severe clinical signs and mortality. Investigations by TEM and NGS revealed a novel parvovirus species, here designated *Phasianus chaphamaparvovirus* 1, within the recently created *Hamaparvovirinae* subfamily and *Chaphamaparvovirus* genus, as the responsible agent for the outbreaks. Analyses of organ tissues from diseased pheasants by in situ hybridization and real-time PCR confirmed the etiology of the outbreaks, as the viral genome was present in the observed lesions. Thus, the present investigation provides a decisive step in establishing the etiology of this pathology in pheasants, more than 50 years after its initial description. Moreover, it highlights the need of more research on this new group of parvoviruses, as it encloses possible important pathogens for different avian species.

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the samples used in this study were collected by Veterinarians during routine farm visits.

CONFLICT OF INTEREST

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

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Cąkała, A. (1966). Szczep wirusa CELO wyosobniony z bazantów. *Medycyna Weterynaryjna*, *22*, 261–264.

Canuti, M., Verhoeven, J. T. P., Munro, H. J., Roul, S., Ojkic, D., Robertson, G. J., Whitney, H. G., Dufour, S. C., & Lang, A. S. (2021). Investigating the diversity and host range of novel parvoviruses from North American ducks using epidemiology, phylogenetics, genome structure, and codon usage analysis. *Viruses*, *13*, https://doi.org/10.3390/v13020193

Cotmore, S. F., Agbandje-McKenna, M., Canuti, M., Chiorini, J. A., Eis-Hubinger, A. M., Hughes, J., Mietzsch, M., Modha, S., Ogliastro, M., Pénzes, J. J., Pintel, D. J., Qiu, J., Soderlund-Venermo, M., Tattersall, P., Tijssen, P., Lefkowitz, E. J., Davison, A. J., Siddell, S. G., Simmonds, P., … Harrach, B. (2019). ICTV virus taxonomy profile: Parvoviridae. *Journal of General Virology*, *100*, 367–368, https://doi.org/10.1099/jgv.0.001212

Day, J. M. (2020). Enteric parvovirus infections of chickens and Turkeys. In: D. E. Swayne, M. Boulianne, C. M. Logue, L. R. McDougald, V. Nair, & D. L. Suarez (Eds.), *Diseases of poultry* (14th edn., pp. 426–445). Hoboken, New Jersey: Wiley-Blackwell.

Dwight, I. A., Coates, P. S., Stoute, S. T., & Pitesky, M. E. (2022). Health surveillance of a potential bridge host: Pathogen exposure risks posed to avian populations augmented with captive-bred pheasants. *Transboundary and Emerging Diseases*, *69*(3), 1095–1107. https://doi.org/10.1111/tbed.14068

Fitzgerald, S. D. (2020). Adenovirus infections. In: D. E. Swayne, M. Boulianne, C. M. Logue, L. R. McDougald, V. Nair, & D. L. Suarez (Eds.), *Diseases of poultry* (14th edn., pp. 321–322). Hoboken, New Jersey: Wiley-Blackwell.

Gelmetti, D., Fabbi, M., Sironi, G., Grilli, G., & Lavazza, A. (1996). Identification of parvovirus-like particles associated with three outbreaks of mortality in young pheasants (*Phasianus colchicus*). *Journal of Veterinary Diagnostic Investigation*, *8*, 108–112, https://doi.org/10.1177/104063879600800118

Grilli, G., Ferrazzi, V., Cislaghi, G., Sironi, G., Lavazza, A., & Daniele, G. (2008). Parvovirus-like hepatitis in grey partridge (*Perdix perdix*): Preliminary observation. In: *Fifty-Seven Western Poultry Disease Conference* (pp. 207–209). Puerto Vallarta, Jalisco, Mexico: WPDC.

Hess, M. (2020). Aviadenovirus infections. In: D. E. Swayne, M. Boulianne, C. M. Logue, L. R. McDougald, V. Nair, & D. L. Suarez (Eds.), *Diseases of poultry* (14th edn., pp. 322–332). Hoboken, New Jersey: Wiley-Blackwell.

Horigan, V., Davies, R. H., Kelly, L. A., Mead, G. C., Irvine, R. M., & Simons, R. R. L. (2014). A qualitative risk assessment of the microbiological risks to consumers from the production and consumption of unevisceratedand eviscerated small game birds in the UK. *Food Control*, *45*, 127–137, https://doi.org/10.1016/j.foodcont.2014.04.040

Kim, H. R., Kwon, Y. K., Jang, I., & Bae, Y. C. (2020). Viral metagenomic analysis of chickens with runting-stunting syndrome in the Republic of Korea. *Virology Journal*, *17*, 1–10, https://doi.org/10.1186/s12985-020-01307-z

Kimball, R. T., Braun, E. L., Zwartjes, P. W., Crowe, T. M., & Ligon, J. D. (1999). A molecular phylogeny of the pheasants and partridges suggests that these lineages are not monophyletic. *Molecular Phylogenetics and Evolution*, *11*, 38–54, https://doi.org/10.1006/mpev.1998.0562

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*, 1547–1549, https://doi.org/10.1093/molbev/msy096

Le, S. Q., & Gascuel, O. (2008). An improved general amino acid replacement matrix. *Molecular Biology and Evolution*, *25*, 1307–1320, https://doi.org/10.1093/molbev/msn067

Liebhart, D., Weissenböck, H., & Hess, M. (2006). In-situ Hybridization for the detection and identification of histomonas meleagridis in tissues. *Journal of Comparative Pathology*, *135*, 237–242, https://doi.org/10.1016/j.jcpa.2006.08.002

Liu, X., Wang, H., Liu, X., Li, Y., Chen, J., Zhang, J., Wang, X., Shen, S., Wang, H., Deng, F., Wang, M., Guan, W., & Hu, Z. (2020). Genomic and transcriptional analyses of novel parvoviruses identified from dead peafowl. *Virology*, *539*, 80–91, https://doi.org/10.1016/j.virol.2019.10.013

Madge, S., McGowan, P. J., & Kirwan, G. M. (2002). *Pheasants, partridges and grouse: A guide to the pheasants, partridges, quails, grouse, guineafowl, buttonquails and sandgrouse of the world* (1st edn.). Princeton, New Jersey: Princeton University Press.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, *17*, 10–12, doi:10.14806/ej.17.1.200

Palya, V. J. (2020). Parvovirus infections of waterfowl. In: D. E. Swayne, M. Boulianne, C. M. Logue, L. R. McDougald, V. Nair, & D. L. Suarez (Eds.), *Diseases of poultry* (14th edn., pp. 474–497). Hoboken, New Jersey: Wiley-Blackwell.

Pénzes, J. J., Söderlund-Venermo, M., Canuti, M., Eis-Hübinger, A. M., Hughes, J., Cotmore, S. F., & Harrach, B. (2020). Reorganizing the family Parvoviridae: A revised taxonomy independent of the canonical approach based on host association. *Archives of Virology*, *165*, 2133–2146, https://doi.org/10.1007/s00705-020-04632-4

Rautenschlein, S., Mahsoub, H. M., Fitzgerald, S. D., & Pierson, F. W. (2020). Hemorrhagic enteritis and related infections. In: D. E. Swayne, M. Boulianne, C. M. Logue, L. R. McDougald, V. Nair, & D. L. Suarez (Eds.), *Diseases of poultry* (14th edn., pp. 339–347). Hoboken, New Jersey: Wiley-Blackwell.

Robertson, D. H., & Hill, D. (1988). *The pheasant: Ecology, management and conservation* (1st edn.). Oxford, England: Wiley-Blackwell.

Rosen, M. N., Hunter, B. F., & Brunetti, O. A. (1965). Preliminary study of an infectious hepatitis in pheasants. *Avian Diseases*, *9*, 382–393.

Swarbrick, O. (1973). Inclusion body hepatitis in pheasants. *Veterinary Record Open*, *93*, 209.

Vibin, J., Chamings, A., Klaassen, M., Bhatta, T. R., & Alexandersen, S. (2020). Metagenomic characterisation of avian parvoviruses and picornaviruses from Australian wild ducks. *Scientific Reports*, *10*, 1–15, https://doi.org/10.1038/s41598-020-69557-z

Wang, Y., Yang, S., Liu, D., Zhou, C., Li, W., Lin, Y., Wang, X., Shen, Q., Wang, H., Li, C., Zong, M., Ding, Y., Song, Q., Deng, X., Qi, D., Zhang, W., & Delwart, E.

(2019). The fecal virome of red-crowned cranes. *Archives of Virology*, *164*, 3–16, https://doi.org/10.1007/s00705-018-4037-x

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