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Histopathological and molecular investigations on partridges (*Perdix perdix*) inoculated with a partridge pegivirus (*Pegivirus alectoris*)

Diploma thesis

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submitted by

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Declaration of Independence

I hereby declare that I have written this thesis independently and have not used any sources or aids other than those indicated. All text passages taken from external sources have been marked. I have carried out the decisive work myself and have indicated all persons involved in the work with their contribution to the work.

This work has not been submitted or published elsewhere.

Vienna, the 08.05.2024 Fatou Louise Geißler

Zusammenfassung

In den letzten Jahren wurden mehrere Enzephalitis-Ausbrüche in Rothuhnfarmen in Frankreich beobachtet. Die betroffenen Rothühner zeigten zentrale nervöse Anzeichen wie Apathie, Torticollis, Ataxie und Prostration. Untersuchungen mittels Next-Generation-Sequenzierung und PCR zeigten virale Sequenzen eines neuartigen Pegivirus - namens *Pegivirus alectoris* (ParPgV) - in Hirnproben. In der vorliegenden Studie wurden Rebhühner intravenös mit filtrierten Homogenaten von ParPgV-PCR-positiven Hirnproben aus Feldausbrüchen infiziert. Die infizierten Vögel zeigten milde klinische Anzeichen, wie vorübergehend reduziertes Flugverhalten. Histologische Untersuchungen ergaben Stauungen, hauptsächlich in Milz, Leber und Niere. Trotz des Fehlens von Läsionen im Hirngewebe zeigte die Echtzeit-RT-PCR-Analyse virale RNA, vor allem im Gehirn, der Milz und in Vollblut. Darüber hinaus bestätigte die In-situ-Hybridisierung das Vorhandensein des Virus im Gehirn, der Milz und der Leber und zeigte positive Signale in Neuronen im Gehirn sowie in Lymphozyten innerhalb der Leber und der Milz. Diese Befunde belegen, dass Rebhühner als empfängliche Wirte für das ParPgV dienen und nicht nur eine lymphotrope, sondern auch eine neurotropische Natur aufweisen. Die vermutete Assoziation zwischen ParPgV und viralen Enzephalitis-Ausbrüchen bei Rothühnern im Feld könnte auf milde neurologische Anzeichen und eine hohe virale Last im Hirngewebe zurückzuführen sein. Horizontale Übertragung konnte noch nicht bestätigt werden, aber das Vorhandensein einer geringen Anzahl positiver Trachea- und Kloakenabstriche deutet auf ein signifikantes Risiko hin. Das Zoonoserisiko des Virus und ökologische Konsequenzen sind noch nicht vollständig geklärt und unterstreichen die Notwendigkeit weiterer Forschung zu Pegivirus bei Rothühnern.

ABSTRACT

In recent years several encephalitis outbreaks were observed in red-legged partridge farms in France. The affected partridges showed central nervous signs, such as apathy, torticollis, ataxia and prostration. Next-generation sequencing, and PCR investigations revealed viral sequences of a novel pegivirus – named *Pegivirus alectoris* (ParPgV) - in brain samples. In the present study, grey partridges were intravenously inoculated with filtrated homogenates from ParPgV PCR-positive brain samples originating from field outbreaks. The inoculated birds showed mild clinical signs, namely transient reduced flying behavior. Histological investigations revealed congestions, primarily in the spleen, liver, and kidney. Despite the absence of lesions in brain tissue, real-time RT-PCR analysis revealed viral RNA, predominantly in the brain, spleen, and whole blood. Furthermore, i*n-situ* hybridization confirmed the presence of the virus in the brain, spleen, and liver, revealing positive signals in neurons in the brain, and in lymphocytes within the liver, and spleen. These findings prove that grey partridges serve as susceptible hosts to the ParPgV and reveal not only lymphotropic nature, but also neural tropism. The suspected association between ParPgV and viral encephalitis outbreaks in red-legged partridges in the field might be due to mild neurological signs and high viral load in brain tissue. Horizontal transmission could not be confirmed yet, but the presence of a small number of positive tracheal and cloacal swabs suggests a significant risk. The virus's zoonotic potential and ecological consequences remain incomplete and highlight the need for further research on pegivirus in red-legged partridges.

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Abbreviation list

ParPgV = Partridge pegivirus

GPgV = Goose pegivirus

HPgV = Human pegivirus

HE = Hematoxylin and eosin

RT-qPCR = reverse transcriptase real-time PCR

ISH = *In-situ* hybridization

HSC = haematopoietic stem cell

PBMCs = peripheral blood mononuclear cells

SPF = specific pathogen free

CSF = cerebrospinal fluid

1. Introduction

The grey partridge (*Perdix perdix*) is a gamebird species widespread in Europe that belongs to the class of Aves, order Galliformes, family Phasianidae and genus *Perdix* (1). Among several others, they are phylogenetically related to red-legged partridges (*Alectoris rufa*). The grey partridge finds its origin in central Europe and Asia, while the red-legged partridge originates from the Iberian Peninsula. Therefore, these species were originally parapatric with central and southern France, and north-west Italy serving as a distributional contact zone. However, both species are sympatric in western Europe, because many red-legged partridges have been released as gamebirds into the UK and France (2). The European Red List Assessment categorizes grey partridges as “Least Concern” in Europe (3). Nevertheless, there has been a decline in population trends, particularly in Austria, where there has been a remarkable decrease of 81% between 1998 and 2020, primarily attributed to habitat loss (3). To address this, many countries intend to hunt and restock grey partridges, so they breed them in captivity and then release them back into the wild (4–6).

The release of gamebirds affects the ecological network and wildlife habitats by competing with local species and environment (7), revealing also gaps of effective disease control (8). In this regard, bird species serve as important natural reservoirs and as amplifying hosts for many viral agents, namely influenza A virus, and flaviviruses such as the Japanese encephalitis virus (9), West Nile virus (WNV), Usutu virus (USUV), and Bagaza virus (BAGV) (10). Therefore, various infectious viral diseases have a higher risk to be introduced in bird populations.

Recently, several encephalitis outbreaks were observed on red-legged partridge farms in France. Clinically, the affected partridges displayed central nervous signs, such as apathy, torticollis, ataxia, and prostration. Next-generation sequencing, and PCR investigations revealed viral sequences of a novel pegivirus - named *Pegivirus alectoris* (ParPgV) - in brain samples of the affected birds. Pegiviruses are enveloped, positive-sense RNA genome viruses that belong to the family *Flaviviridae* and have been detected in a wide range of mammalian species but are considered non-pathogenic (11). However, it has been reported that Theiler’s disease in horses is associated with infection with members of species *Pegivirus equi* (12). Non-mammalian pegiviruses have been reported recently in China, in geese, passerines, and finches, and in Australia in mynas (13,14). In geese, it was reported that pegivirus infection resulted in growth retardation, and the virus demonstrated high

lymphotropism in infected goslings. However, no clinical signs were reported in passerines, finches, and mynas, where the virus was detected during routine monitoring (9,13,14).

In this study, an experimental infection in grey partridges was conducted by inoculating them intravenously with filtrated homogenates from ParPgV PCR-positive brain samples originated from field outbreaks, followed by daily monitoring of birds' clinical status, weekly sampling, and postmortem investigation of selected organs by histology and PCR techniques.

Hypothesis:

1. A novel pegivirus (ParPgV) is the responsible agent of field outbreaks of viral encephalitis in red-legged partridges.
2. Grey partridges, which are more prevalent in central Europe, are also a susceptible host to the ParPgV.
3. In addition to blood and lymphoid organs, the ParPgV can also be detected in the brain of infected partridges.
4. Infection with ParPgV leads to neurological clinical signs and histological lesions in the central nervous system (CNS).

Objectives:

a) Develop a real-time RT-PCR assay for prompt viral load detection in target organs, enhancing upon existing conventional RT-PCR methods.
b) Conduct comparative analyses of conventional and real-time RT-PCR techniques on target organ samples to assess ParPgV detection efficacy.
c) Utilize real-time RT-qPCR to ascertain viral RNA presence and semi-quantification across diverse target organs.
d) Establish an *in-situ* hybridization methodology for detecting ParPgV genomic RNA in PCR-positive histological organ samples.
e) Perform histological examinations on postmortem organ samples, contrasting histological profiles between ParPgV-infected and uninfected avian subjects.
f) Investigate viral RNA distribution and tropism via *in-situ* hybridization on histological organ samples exhibiting pathological alterations or positive real-time RT-qPCR results.

2. Literature review

2.1. Taxonomy and biology

The grey partridge (*Perdix perdix*) is a gamebird species widespread in Europe that belongs to the class of Aves, order Galliformes, family *Phasianidae* and genus *Perdix* (1). The *Phasianidae* family includes several game-bird species, and can be divided into three subfamilies: *Phasianinae*, *Pavoninae,* and *Rollulinae* (1). The first group includes pheasants, tragopans, junglefowl, and peafowl, while the second one contains partridges, Old World quails, and francolins (15). Grey partridges originally lived in short steppe and open grassland but are nowadays mainly found on farmland (16). They colonize most parts of Europe and reach the eastern parts of Mongolia in Asia (16). Due to habitat loss and consequently reduced breeding success, their populations numbers are decreasing (16).

Pegivirus belongs to the family *Flaviviridae* and is characterized by enveloped virions enclosing a single-stranded, positive sense RNA genome, closely related to the Hepatitis C virus (17). The genera *Hepacivirus*, *Orthoflavivirus*, and *Pestivirus* also belong to the family *Flaviviridae*, but show differences in their genome organization (18). Pegiviruses have been found in various mammalian species, including humans, non-human primates, pig, horses, as well as various rodent and bat species (18). There is limited information on the potential of pegivirus to transmit between different host species (18).

2.2. Epidemiology

Pegiviruses have been found in many mammals, including humans, non-human primates, bats, horses, pigs, cats, rodents, and dolphins (9,19,20). Cases of pegivirus detection in non-mammalian species are very rare and were only reported recently in geese, passerines, finches, and mynas so far (9,13). Therefore, knowledge on the zoonotic potential and ecological impact of the virus is largely uncovered, however seen as of great importance since game birds can have significant impact when introduced into different habitats for hunting and restocking purposes, due to their migratory behavior and close interactions with native bird species (9).

In humans, pegiviruses were found in the saliva and serum of healthy individuals and demonstrated robust *in vivo* replication (21). Furthermore, the viraemia caused by human

pegivirus (HPgV-1) can persist for several decades by avoiding immune recognition and T-cell immune activation (21). Nonetheless, the virus has been linked to medical conditions including the development of lymphoma, and the occurrence of fatal brain leukocyte encephalitis (21). HPgV-1 RNA was found in various blood mononuclear cells, such as T-lymphocytes, B-lymphocytes, NK cells, and monocytes (21). Therefore, the virus shows a wide-ranging cellular tropism for both lymphoid and myeloid cells, with a preference for replication in the bone marrow and spleen. It does so without causing a cytopathic effect (21). The virus has been generated in *in-vitro* PBMCs culture systems, but its production has been notably restricted (21). This implies that haematopoietic stem cells (HSC) could be the principal target of HPgV-1 infection and indicates that the virus continues to endure and replicate throughout lymphocyte maturation and subsequent stages (21). In geese, the goose pegivirus (GPgV) successfully replicated in geese embryos and goose embryo fibroblasts (GEF's) and demonstrated a pronounced affinity for lymphoid tissues in experimentally infected goslings, with the spleen and thymus exhibiting the highest viral loads (14).

In humans, pegivirus is a blood-borne virus and is transmitted by percutaneous injuries, blood infusions, sexual contact, and from mother to child (21). HPgV viremia rates are elevated in injecting drug users and in hemophiliacs exposed to coagulation factor concentrates that have not been treated to inactivate the virus, suggesting an effective mode of transmission via parenteral routes (18,21). Even though, horizontal or vertical transmissions have not been confirmed in geese so far, a relatively high viral load was found in small and large intestine, but it was limited in fecal samples. However, these data do not exclude the possibility of horizontal transmission via virus-contaminated feed and water (14).

3. Animals, Material and Methods

3.1. Virus and Experimental design

3.1.1. Virus inoculum

ParPgV PCR-positive brain samples from a field outbreak in red-legged partridges, identified as 22-02813/LH234A, were homogenized (ULTRA TURRAX T 10 basic, IKA, Germany) in phosphate buffered saline (PBS) (20% wt/vol), containing 1 mg/ml streptomycin and 100,000 IU/ml penicillin. Then, the homogenates were clarified by centrifugation at 2000 x g for 10 min. The supernatant was collected, filtered through a 0.2 µm pore-sized filter (VWR, Vienna, Austria) and the filtrated material was used to inoculate the birds.

3.1.1. Experimental design

Ten 5-month-old grey partridges were locally obtained from a commercial farm (Erich Mühlböck, Natternbach, Austria), individually ringed, and divided into two groups, namely group A and group B. Group A consisted of five partridges – partridges (par) 4,6, 8, 9, and 10 – which were inoculated, while group B consisted of three partridges – par 1-3 – that were used as negative controls. The groups were kept separated in different rooms, in floor pens on wood shavings and light was set at a 12h:12h light/dark cycle. On the first day of the experiment, group A was inoculated intravenously with 0.2 ml of the virus filtrate, while group B was inoculated intravenously with 0.2 ml of PBS (**Table 1**). Following inoculation, birds were clinically monitored every day. Birds were sampled for blood pre-inoculation and weekly post-inoculation. Additionally, upper respiratory tract and cloacal swaps were collected every 3 to 4 days. At day 5 post-inoculation (dpi), par 1 from group B died. At 14 dpi, two birds from group A (par 4 and 9), and one from group B (par 3) were euthanized for post-mortem assessment and sampling. The remaining birds were euthanized at 21 dpi (**Table 1**). Organ samples were then taken from the dead animals, which included brain, heart, kidney, spleen, and liver. All procedures were previously discussed and approved by the institutional ethics and welfare committee and the national authority according to §§26ff. of Animal Experiments Act, Tierversuchsgesetz 2012 – TVG 20212 (license number: GZ. 2022-0.713.294).

3.2. Materials and Methods

3.2.1. Histology

Samples of brain, spleen, liver, heart, and kidney were fixed in 4% neutral buffered formalin (SAV LP GmbH, Flintsbach, Germany) during necropsy, and then embedded in paraffin for microscopic examination. Following, the paraffin-embedded samples were cut in a microtome (Microm, HM 360; Microm Laborgeräte GmbH, Walldorf, Germany) into 5-µm thick sections. These were then mounted onto a glass slide and incubated overnight at 40°C. The slides were stained with haematoxylin and eosin (H&E).

3.2.2. Molecular investigation

3.2.2.1. RNA extraction and RT-PCR investigation

Total RNA extraction was performed from brain, spleen, liver, heart, kidney, whole blood, and swab specimens using the RNeasy Plus Kit (QIAGEN, Vienna, Austria), following the manufacturer's protocols. The eluted total RNA was obtained in a final volume of 50 µl using the supplied elution buffer. Subsequently, specific primers designed for the ParPgV NS3 gene sequence, previously identified through next-generation sequencing analysis of outbreak samples (**Table *2***), were employed to probe the experimental animal samples for the presence of ParPgV. Reverse transcriptase (RT)-PCR was carried out using the OneStep RT-PCR kit (QIAGEN), following the manufacturer's guidelines, with a final primer concentration of 500 nM for both the forward and reverse primers. Negative PCR controls were systematically incorporated into all RT-PCR reactions to monitor for potential contaminations. The RT-PCR cyclic conditions are shown in **Table 3**. The resulting PCR products were subjected to gel electrophoresis using a 1.5% (w/v) Tris acetate-EDTA-agarose gel, run at 100 V for 60 minutes, stained with GelRed® (Biotium, Vienna, Austria), and visualized under ultraviolet light using the BioRad Universal Hood II (Bio-Rad Laboratories, Hercules, CA).

3.2.2.2. Establishment of a real-time RT-PCR and subsequent sample investigation

To quantify the pegivirus genome in the organ samples, we established a real-time reverse transcription PCR (RT-qPCR) assay. We conducted an initial RT-qPCR run to determine the optimal primer concentrations for both the upstream and downstream primers (**Table *2***). Our aim was to identify primer concentrations that would yield a robust assay with minimal

nonspecific amplification or primer-dimer formation. Additionally, we analyzed the threshold cycle (Cq) values and constructed standard curves to ensure efficient amplification, facilitating the generation of reliable and reproducible data.

We experimented with a range of primer concentrations, spanning from 200 to 600 nM, in combination with five tenfold dilution series of the target RNA sequence derived from a brain sample from field outbreak 22-2813/LH234A, down to a dilution of 10^{-4}. For each primer concentration, we included a no-template control (NTC) reaction. The RT-qPCR reactions were conducted using the Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix (Agilent Technologies, Vienna, Austria) following the manufacturer's instructions, employing an Agilent AriaMX thermocycler (Agilent Technologies). The cyclic conditions are shown in **Table 4**. Each 20-µl reaction contained 2-µl of the template RNA. The optimal primer concentrations were defined as those resulting in the lowest Cq values and adequate fluorescence signals for a given target concentration.

Once the optimal primer concentrations were determined, we empirically determined the optimal concentration of the probe (**Table 2**). We tested a range of probe concentrations, varying from 100 to 500 nM, in a master mix alongside the previously established primer concentrations. Again, we used a five tenfold dilution series of the target RNA sequence and included NTC reactions. The RT-qPCR reactions were carried out using the Brilliant III Ultra-Fast QRT-PCR Master Mix (Agilent Technologies), following the manufacturer's recommendations, in an Agilent AriaMX thermocycler (Agilent Technologies). As before, each 20-µl reaction contained 2-µl of the template RNA. The RT-qPCR cyclic conditions are shown in **Table 3**. Subsequently, we calculated amplification efficiencies using Agilent AriaMX software version 1.7 (Agilent Technologies) and generated graphical representations in the form of linear regression curves and amplification plots.

Samples of brain, spleen, liver, kidney, heart, whole blood, and swabs were then investigated by the established RT-qPCR, using forward and reverse primer final concentration of 500 nM, each and final probe concentration of 200 nM. The thermocycler, mastermix kit, and cyclic conditions followed the ones previously described for the probe concentration optimization.

3.2.3. *In-situ* hybridization (ISH)

In-situ hybridization (ISH) was performed on paraffin-embedded experimental samples of brain, spleen, kidney, liver, and heart from par 6, 8, 9, and 10. The procedure followed the protocol previously published by Fana Kidane et al (22), but utilizing two digoxigenin-labeled antisense DNA probes, simultaneously, targeting the 5' UTR and NS4B viral genomic regions of the ParPgV (**Table 2**) in a concentration of 100 ng/ml of hybridizations solution. A no-probe control, and a heterologous probe control were used, in which PBS was used instead in the former, and a probe targeting the parasite *Histomonas meleagridis* was used in the latter (23). The chromogenic detection reaction took place overnight for a total of 20h and monitored in between.

4. Results

4.1. Histopathology

Histological investigation revealed changes mainly in kidney, liver, and spleen.

Mild to severe congestions were observed in the kidney in all inoculated birds from group A, characterized by erythrocyte infiltration (**Figure 1a-b**). Additionally, par 9 displayed the described alterations along with an infiltration of basophilic cells, devoid of any observable surrounding connecting tissue (**Figure 1c**).

In liver, except for par 10, all the others displayed varying degrees of congestion. These changes were characterized by the infiltration of erythrocytes into the tissue as presented in **Figure 1d**.

Among group A, par 4, 6, and 8 exhibited varying degrees of splenic congestion, ranging from mild to severe, while the remaining birds did not display any observable alterations. The spleen from par 8 was notable for having a basophilic nodule, while another presented an encapsulation of connective tissue *(***Figure 1e-f***).*

In the organ samples collected at 21 dpi, multiple basophilic nodules were observed in the duodenum of two out of the three remaining birds in group A, with one of them also presenting mild hemorrhagic changes (**Figure 2**). Additionally, the bird remaining in group B (control), also exhibited these nodules found in group A.

The remaining investigated organs did not show any changes during histological investigation.

4.2. Conventional and real-time RT-PCR investigations

After inoculation, birds were daily monitored, and tracheal swabs, cloacal swabs, and whole blood samples were collected to be investigated by PCR (**Table 4**). Tracheal swabs investigation revealed positive results for par 8, and 10 at 21 dpi with Ct values of 26.54 and 31.72, respectively, by real-time RT-qPCR.

Par 10 was tested positive in cloacal swabs at both 17 (Ct 30.63) and 21 (Ct 32.28) dpi, while par 4 displayed positive results with a Ct value of 33.19 only at 10 dpi and subsequently turned negative.

Blood samples were taken at 7, 14, and 21 dpi. All examinations showed positive outcomes with Ct values ranging from 21.94 to 35.02 among all partridges, except for par 6, and par 10 that were registered negative at 14 and 21 dpi (**Figure 3**).

The post-mortem molecular analysis was carried out on brain, spleen, liver, kidney, and heart samples as shown in **Figure 4** and **Table 5**.

The investigation showed positive results in all five partridges for brain and spleen samples in RT-qPCR and conventional RT-PCR, except for par 4 that was tested negative for both samples in the RT-PCR analysis. The Ct values for brain ranged from 22.27 to 28.25, while spleen Ct values ranged from 25.49 to 29.56.

In the liver, par 10 displayed positive results in RT-PCR and RT-qPCR with a Ct value of 24.64, whereas par 8 exhibited positivity only in the RT-qPCR analysis with a Ct value of 23.89.

Par 8, 9, and 10 were tested positive in the RT-qPCR, conducted on kidney tissue with Ct values of 34.74, 34.68, and 31.02, respectively, while all remaining outcomes of the conventional RT-PCR and RT-qPCR were indicated negative.

All five partridges were evaluated as negative for the heart samples.

4.3. *In-situ* hybridization

In-situ hybridization confirmed viral presence in brain, liver, and spleen of the inoculated birds as presented in **Figure 5**.

Among group A, par 6, 8 and 10 displayed a small number of positive signals in brain samples, predominantly found associated with neurons in the cerebrum (**Figure 5a-b**). The virus was sparsely distributed throughout the brain tissue, characterized by scattered dark violet signals.

As illustrated in **Figure 5c**, the virus was detected across a broad area of the liver in par 8 and 10, indicating its presence within the cytoplasm of hepatocytes. Furthermore, the signals were observed in an area of lymphocyte infiltration in par 8.

Par 8 and 9 were tested positive for the virus in spleen samples, where the signals accumulated in and around lymphocytes (**Figure 5d).**

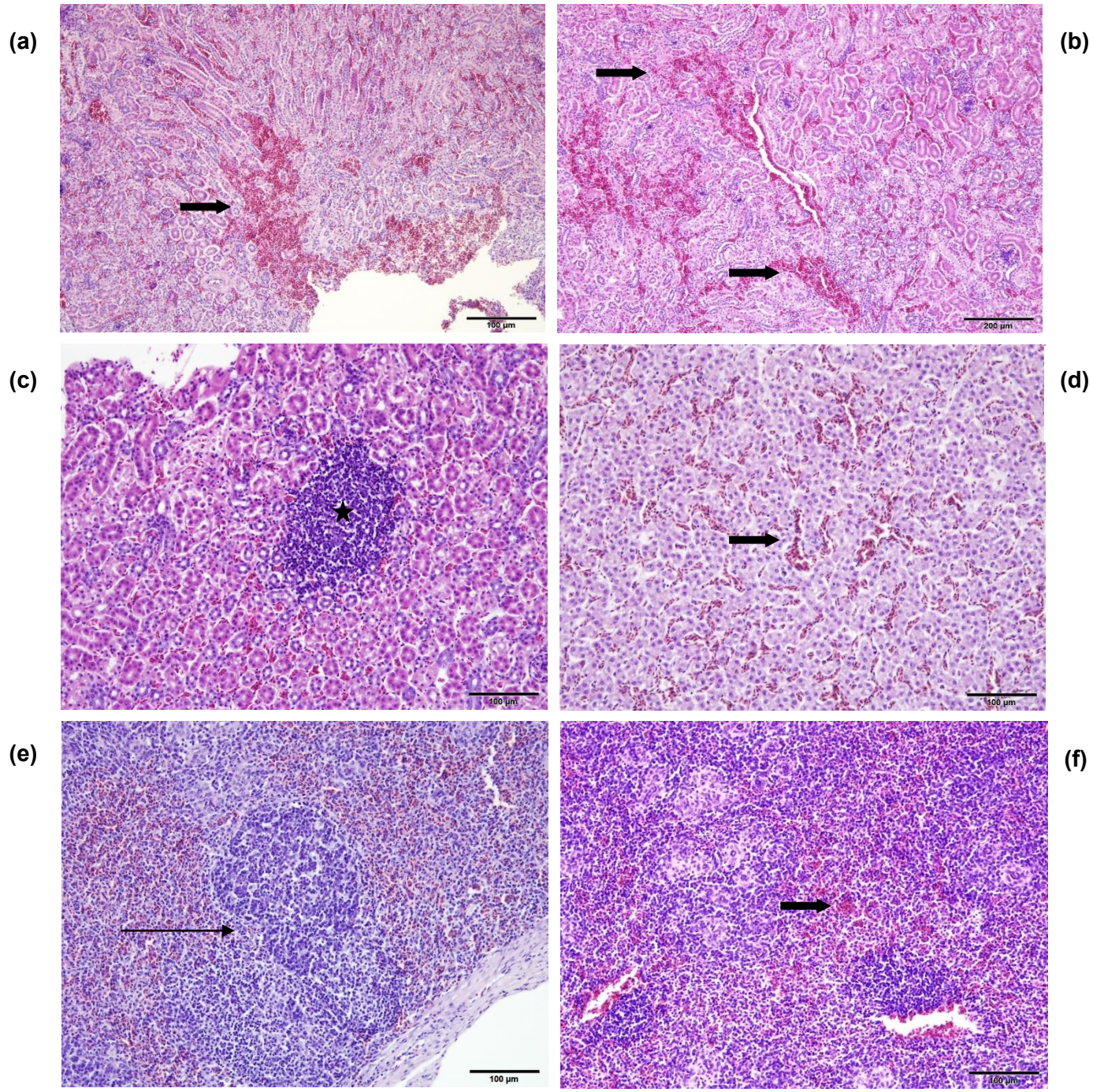

Figure 1: Histopathological investigation.

ParPgV-inoculated partridges presented various degrees of kidney congestions (➔): characterized by erythrocyte infiltration (a-b) and presented an additional infiltration of basophilic cells (⋆), devoid of any observable surrounding connecting tissue (c). The histopathology examination revealed mild liver congestion characterized by erythrocyte infiltration (d), and the presence of basophilic encapsulation (→) within the parenchymal tissue in the spleen (e). The spleen displayed various degrees of congestions (f).

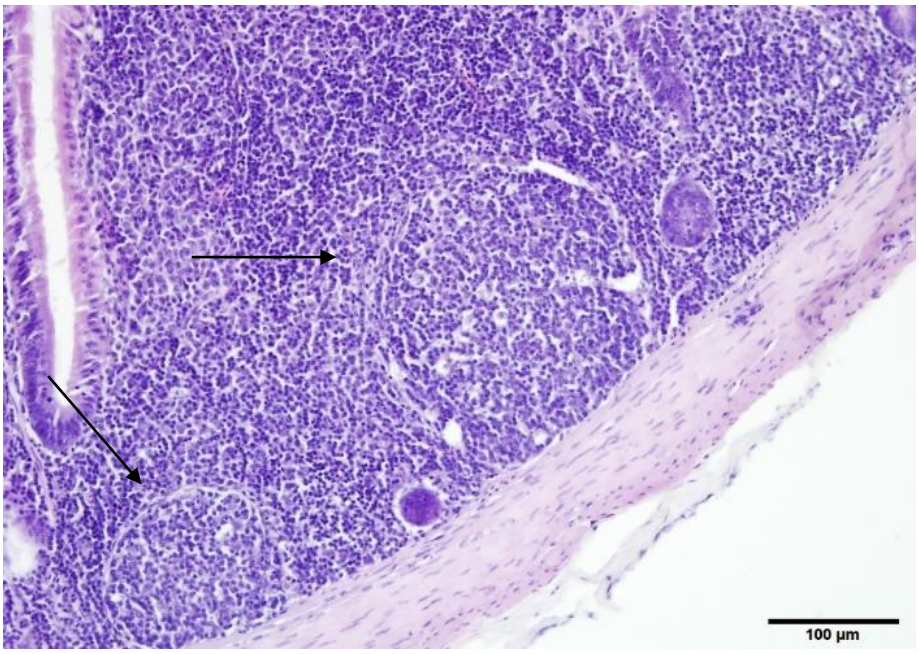

Figure 2: Histopathological investigation of intestine

Partridge 10 displayed multiple basophilic nodules enclosed within a capsule of connecting tissue (→).

Table 1: Experimental design of the animal trial in grey partridges (*Perdix perdix*).

groups	bird type	age of birds	no. of birds	route of inoculation	blood sampling (dpi[a])	swab sampling (dpi)
A	Grey partridge (*Perdix perdix*)	5 months-old	5	i.v. [b]	0, 7, 14, 21[c]	0, 3, 7, 10, 14, 21[c]
B			3	n. a.[d]		

[a] days post-inoculation.
[b] intravenous.
[c] the trial was terminated at 21 dpi.
[d] not applicable.

Table 2: **List of primers and probes used for conventional RT-PCR, real-time RT-qPCR, and in-situ hybridization investigations.**

Targeted gene	Primer/probe	Sequence (5' → 3')	Method
NS3	Pegi260-F[a]	AGC ATC CAA GAC TCC TTG GC	PCR
	Pegi260-R[b]	GCGGCTTTGGTGAACTACAC	
5' UTR	ParPgV_5UTR-F	AGT GCT TAT AAA CTC AGT ACC CTG G	Real-time RT-PCR
	ParPgV_5UTR-R	GAT GCT TTG TCA AAT CGC GGA TAG	
	ParPgV_5UTR-P	TTC CCC AAG GCG GCA ACG GGC TAG GC	
	Pegi_150bp_ISH-1	CGTGTCACGAATATTTGAGTCATGGGACCCCTAAAGGGGTT	*In-situ* hybridization
NS4B	PegiAC_NS4B_ISH	AAAGGGCCTCGACCGCCTTAATGGTGTGGCGGACAGCCTA	

[a] Forward primer.
[b] Reverse primer.
[c] Probe.

Table 3: **PCR cyclic conditions for the different PCR investigations performed.**

Method	Step	Cycles	Duration of cycle	Temperature
RT-PCR	Reverse transcriptase	1	30 min	50°C
	Hot start	1	15 min	95°C
	Amplification	35	30 sec	95°C
			30 sec	59°C
			1 min	72°C
	Final extension	1	10 min	72°C
SYBR® Green RT-qPCR	Reverse transcriptase	1	15 min	50°C
	Hot start	1	3 min	95°C
	Amplification	40	5 sec	95°C
			20 sec	60°C
	Melt	1	1 min	95°C
			30 sec	55°C
			30 sec	95°C
Real-time RT-qPCR	Reverse transcriptase	1	15 min	50°C
	Hot start	1	3 min	95°C
	Amplification	40	5 sec	95°C
			20 sec	60°C

Table 4: Summary of RT-qPCR results of tracheal swabs, cloacal swabs, and whole blood of virus-inoculated partridges from group A (par 4, 6, 8, 9, and 10)

		4 dpi [a]	7 dpi	10 dpi	14 dpi	17 dpi	21 dpi
Par 4	tracheal swabs	neg [b]	neg	neg	neg		
	cloacal swabs	neg	neg	pos [c] /Ct 33.19	neg	n.a [d]	
	whole blood	n.d[e]	pos/Ct 32.29	n.d	pos/Ct 29.80		
Par 6	tracheal swabs	neg	neg	neg	neg	neg	neg
	cloacal swabs	neg	neg	neg	neg	neg	neg
	whole blood	n.d	pos/Ct 36.18	n.d	neg	n.d	neg
Par 8	tracheal swabs	neg	neg	neg	neg	neg	pos/Ct 26.54
	cloacal swabs	neg	neg	neg	neg	neg	neg
	whole blood	n.d	pos/Ct 21.94	n.d	pos/Ct 35.02	n.d	pos/Ct 26.42
Par 9	tracheal swabs	neg	neg	neg	neg		
	cloacal swabs	neg	neg	neg	neg	n.a [d]	
	whole blood	n.d	neg	n.d	neg		
Par 10	tracheal swabs	neg	neg	neg	neg	neg	pos/Ct 31.72
	cloacal swabs	neg	neg	neg	neg	pos/Ct 30.63	pos/Ct 32.38
	whole blood	n.d	pos/Ct 25.91	n.d	neg		neg

[a] days post-inoculation.
[b] negative.
[c] positive/threshold cycle.
[d] not applicable.
[e] not done.

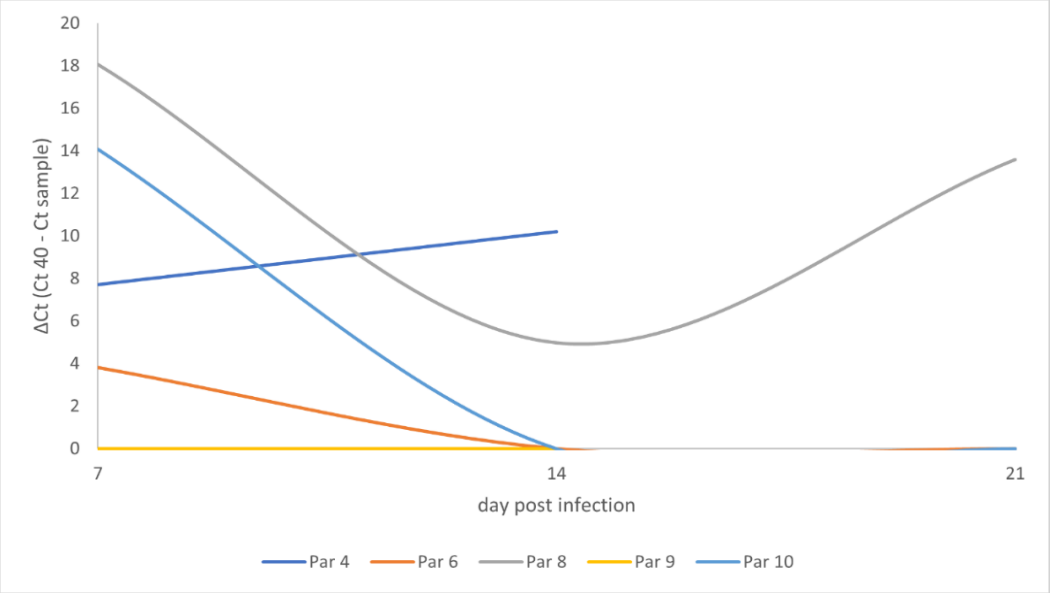

Figure 3: RT-qPCR results for whole blood samples in partridge 4, 6, 8, 9, and 10, at 7, 14, and 21 dpi. Results are presented in ΔCt's, corresponding to a difference between the total number of PCR cycles – 40 – and the sample Ct, to allow a better semi quantitative interpretation of the viral load.

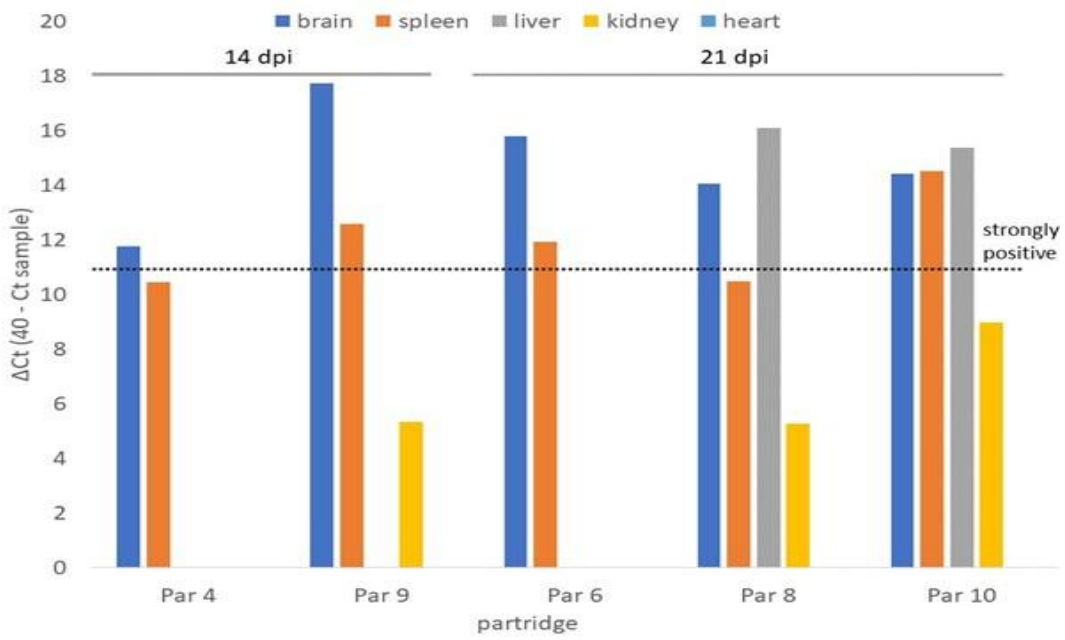

Figure 4: RT-qPCR results of brain, spleen, liver, kidney, and heart from ParPgV-inoculated partridges from group A. Partridges 4 and 9 were euthanized at 14 day post-infection, while partridge 6, 8, and 10 were euthanized at 21 days post-infection. Results are presented in ΔCt's, corresponding to a difference between the total number of PCR cycles – 40 – and the sample Ct, to allow a better semi quantitative interpretation of the viral load in the target organs. Results with a Ct < 29 are considered strongly positive.

Table 5: Summary of histology, in-situ hybridization, RT-PCR, and RT-qPCR; results in brain, spleen, liver, kidney of virus-inoculated partridges from group A (Par 4, 6, 8, 9, and 10)

		Par 4	Par 6	Par 8	Par 9	Par 10
Brain	Histology	no lesions	no lesions	no lesions	no lesions	no lesions
	ISH[a]		pos[d]	pos	neg[e]	pos
	RT-PCR[b]	neg	pos	pos	pos	pos
	RT-qPCR[c]	pos/Ct 28.25[f]	pos/Ct 24.19	pos/Ct 25.93	pos/Ct 22.27	pos/Ct 25.57
Spleen	Histology	severe congestion	severe congestion	mild congestion, basophilic nodule	no lesions	no lesions
	ISH		neg	pos	pos	neg
	RT-PCR	neg	pos	pos	pos	pos
	RT-qPCR	pos/Ct 29.56	pos/Ct 28.07	pos/Ct 29.50	pos/Ct 27.41	pos/Ct 25.49
Liver	Histology	mild congestion	mild congestion	mild congestion	mild congestion	no lesions
	ISH		neg	pos	neg	pos
	RT-PCR	neg	neg	neg	neg	pos
	RT-qPCR	neg	neg	pos/Ct 23.89	neg	pos/Ct 24.64
Kidney	Histology	severe congestion	congestion	mild congestion	congestion, basophil nodular encapsulation	congestion
	ISH		neg	neg	neg	neg
	RT-PCR	neg	neg	neg	neg	neg
	RT-qPCR	neg	neg	pos/Ct 34.74	pos/Ct 34.68	pos/Ct 31.02
Heart	Histology	no lesions	no lesions	no lesions	no lesions	no lesions
	ISH		neg	neg	neg	neg
	RT-PCR	neg	neg	neg	neg	neg
	RT-qPCR	neg	neg	neg	neg	neg

[a] *In-situ* hybridization.
[b] Reverse-transcription polymerase chain reaction.
[c] Reverse transcription-quantitative polymerase chain reaction.
[d] Negative.
[e] Positive
[f] Threshold cycle.

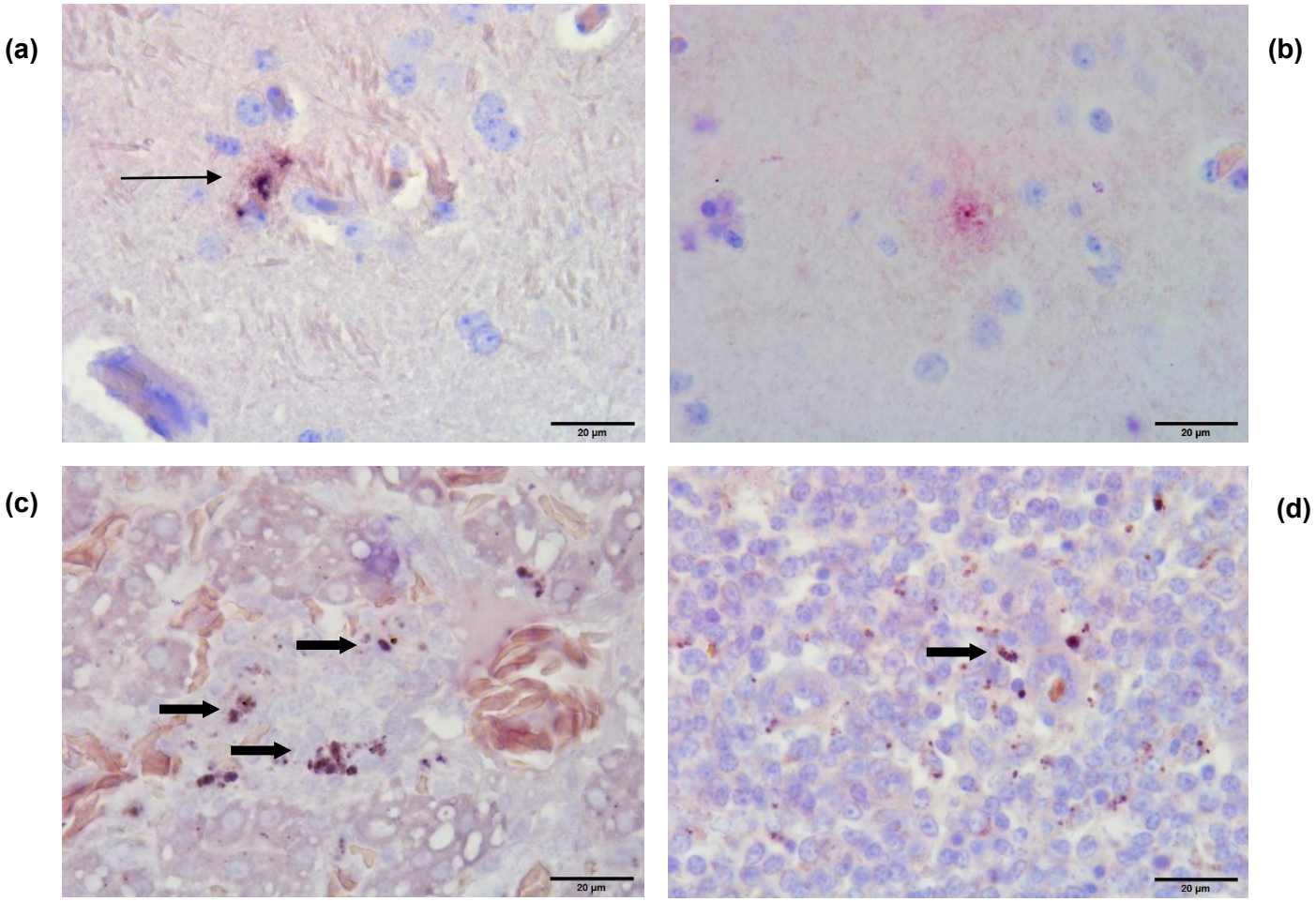

Figure 5: In-situ hybridization of brain, liver and spleen

In-situ hybridization revealed the presence of the virus in neurons (→) and in the neuropil of the brain (a-b), as well as in lymphocytes (➔) within liver tissue (c). In spleen, the virus predominantly accumulated in and around lymphocytes (d).

5. Discussion

Viruses belonging to the genus *Pegivirus*, family *Flaviviridae*, mostly infect mammals, including humans, non-human primates, bats, horses, pigs, cats, rodents, and dolphins (9,14). In a recent study, pegivirus was detected in geese (*Anser cygnoides*) making it the first identified non-mammalian host species, suggesting that pegiviruses are not restricted to mammals and may, therefore, be present in additional groups of vertebrates (14). Although, the virus is not considered pathogenic, it is capable to cause persistent infections in its host (11,21). However, the knowledge on pathogenicity, cell tropism, transmission, biology, and epidemiology are, so far, very limited. The HPgV-1 can cause a viraemia persisting over decades through evasion of immune recognition and T-cell immune activation (21). Despite the extended existence, the virus has been linked to several conditions, such as fatal brain leukocyte encephalitis, detected across a range of mononuclear blood cells (21).

Both mammals and birds are recognized as hosts for flaviviruses, exhibiting a diverse clinical picture that vary from asymptomatic to fatal conditions such as hemorrhagic fever or dysfunction of the central nervous system (11). Due to limited availability of experimental animal models and *in-vitro* culture systems, the epidemiology of pegivirus has been mostly unknown (24). However, in the Sichuan Province and the Congqing municipality of China, the first identified non-mammalian host species was discovered among geese and was identified as a sister clade to the mammalian pegivirus (14). Moreover, the pegivirus was detected in various wild birds in China, and in mynas in Australia, expanding the host range of pegivirus to include avian species (9). This emphasizes the significance of birds serving as reservoirs or amplifying hosts for several emerging or re-emerging viral pathogens (9,13). Despite the limited information of the potential transmission between different host species, it was possible to trace back a common ancestor to 312 million years ago, before the divergence of mammals and birds (9). This suggests an ancient origin of the genus *Pegivirus* (9), and confirms that grey partridges in central Europe can serve as susceptible hosts for ParPgV.

In this study, a novel pegivirus responsible for several encephalitis outbreaks in red-legged partridges in France, characterized by neurological signs such as apathy and torticollis, was inoculated in grey partridges. The birds did not present severe clinical signs, and only transient reduced flying behavior was observed. The histological investigation revealed that the most affected organs were spleen, liver, and kidney. The organs showing the highest

viral load detected through RT-qPCR were spleen and brain. Further analysis with *in-situ* hybridization revealed positive signals in these organs, as well as in the liver.

One of the most remarkable findings was that brain tissue did not show any changes in the histological examination and therefore, did not present any signs of encephalitis. However, in all partridges the virus was detected in the brain samples with a ranging Ct value from 22.27 to 28.25. Previous cases involving HPgV-1 have demonstrated that under certain circumstances the virus can be detected in the brain. The presence of virus in the brain may result from compromised blood-brain barrier, which is possible in patients suffering from encephalitis (25). While the HPgV-1 was described to cause fatal leukocyte encephalitis characterized by lymphocytic infiltration and gliosis (21), the partridges inoculated in our studies did not show any signs of inflammation or damages to brain tissue. Moreover, HPgV-1 showed neurotropism with specific infection in astrocytes and microglial cells (21). In our studies, we observed high Ct values from the RT-qPCR analysis in the brain, yet only a small number of positive signals were detected through *in-situ* hybridization in neurons of the cerebrum. However, ISH and histological examinations evaluate only a sagittal section of the brain, requiring multiple sections to obtain a comprehensive overview of the entire organ. In contrast, RT-qPCR analysis is more sensitive, due to homogenized larger organ pieces, thereby offering a broader perspective of the investigated organ. Furthermore, exponential amplification of RNA generates a larger number of copies, which can be detected through fluorescent probes, significantly enhancing sensitivity and allowing a more precise identification of positive signals compared to ISH.

Neurological signs can also be induced by other members of the Flaviviridae family, including West Nile virus, Usutu virus, and Bagaza virus (10). In grey partridges, an experimental infection with Bagaza virus caused a progressive neurological disorder, including signs such as weakness, apathy, unresponsiveness, ruffled feathers, ataxia and prostration (26). Only a few exhibited severe neurological signs such as partial or complete paralysis, along with ocular lesions (26). Given the absence of evident neurological signs in our experiment, the lack of histological lesions could be a contributing factor. Nonetheless, a direct link between pegivirus and the observed clinical signs remains possible, due to the notable viral load detected in brain tissue. An infection with ParPgV might lead to a progressive neurological disorder, only detectable through PCR examinations in early stages. Furthermore, the co-infection of pegivirus with other viruses should be taken into consideration, as it may

contribute to the development of disease, secondary infection or inflammatory responses (24).

Goslings infected with GPgV showed visible lesions in thymus, spleen, and intestine, that included serious splenectasis and enlarged, red thymus. Additionally, thymus and spleen displayed signs of congestion, and high levels of lymphotropism (14,24). In our studies, congestion was observed in spleen, kidney, and liver, with an additional bird showing hemorrhagic changes in the kidney. The PCR investigation yielded positive results in spleen, with Ct values ranging from 25.49 to 29.56, and in kidney samples from three out of five partridges, with Ct values from 31.02 to 34.74. Likewise, the spleen and thymus, as mentioned in previous studies on GPgV, were identified for having the highest viral load (11,14) Furthermore, the RNA of HPgV-1 was predominantly detected in bone marrow and spleen, indicating that the primary target of the virus may be hematopoietic stem cells (HSC) (21). In our studies, *in-situ* hybridization detected viral presence in spleen samples from two out of four partridges, revealing virus accumulation in and around lymphocytes. Previous research on HPgV-1 has demonstrated that the virus can be found in various blood mononuclear cells, including T- and B-lymphocytes, highlighting its tropism for lymphoid cells and suggesting a replication in spleen by enduring throughout maturation and subsequent stages (21). Therefore, our study supports the notion that the spleen primarily serves as a reservoir for the virus's invasion, emphasizing lymphatic organs being a main target of the infection.

In tissue samples from pegivirus-infected pigs, the liver showed a mild, portal-accentuated, and lymphohistiocytic hepatitis (27). Wild birds infected with Bagaza virus displayed liver congestions, focal hepatocyte necrosis, and a moderate mononuclear infiltration (28). In studies targeting grey partridges with Bagaza virus, the liver was also impacted; however, demonstrated the lowest load of viral RNA compared to kidney, heart, and brain (26,28). In our studies, only two out of five partridges displayed positive results through RT-qPCR with a Ct value of 23.89 in partridge 8 and 24.64 in partridge 10. However, histological examination revealed mild liver congestions in all birds, except for partridge 10. Despite the high viral load detected through RT-qPCR in the liver, this bird did not show any lesions. This might be connected to the negative results in blood samples of partridge 10 at 14 and 21 dpi, while partridge 8 was tested positive for the virus in blood at 7, 14, and 21 dpi. It is also important to note that typical postmortem changes in liver can involve congestions caused by venous blood pooling and dilation of blood vessels. Consequently, these alterations may also be

observed in samples where no viral load was detected and can therefore be a result of euthanasia. Interestingly, the identification of the HPgV-1 virus initially stemmed from patients experiencing acute or chronic non-A-E hepatitis, leading to its primary classification as a hepatotropic virus (21). Further investigation revealed that HPgV-1 RNA predominantly occurred in lymphocytes, with minimal to no presence in liver samples (21). This indicates that HPgV-1 is more likely lymphotropic rather than hepatotropic (21). Using *in-situ* hybridization, ParPgV was detected within the cytoplasm of hepatocytes in the liver of par 8, and 10. Additionally, signals were observed in areas of lymphocyte infiltration in par 8 of the liver, suggesting a predominantly localization of the virus within lymphocytes. Therefore, the infection of lymphocytes in both, liver and spleen, indicates the virus to be rather lymphotropic.

In previous studies, Usutu virus has been described to cause inflammation in heart tissue not only in chickens, but also in wild bird populations in Europe, showing signs of myocarditis with lymphocytic infiltrates in the heart (29). Furthermore, other members of the *Flaviviridae* family, like the Bagaza virus, displayed a high RNA viral load in heart tissue, particularly in early stages of infection (3-6 dpi) (26). Histology, ISH, and PCR examinations of heart tissue did not reveal viral presence in our case. It's noteworthy that in previous studies on pegivirus, the examination for viral presence did not include heart samples. Consequently, there is no indication that the heart serves as a target organ for ParPgV.

In this study, basophilic nodules were found in spleen, liver, kidney, and intestinal tissue. However, they were also found in the negative control, which leads to the conclusion to be physiological findings of immunological tissue in partridges. Par 10 showed an encapsulation surrounded by connective tissue in the intestine which could be an additional finding of a parasitic presence but is not relevant to our studies. Another bird from the negative control group died early during the animal trial, exhibiting hemorrhagic alterations in the kidney. These findings were neglected, as they might be due to the absence of specific-pathogens-free (SPF) animals in this study.

The transmission of avian pegiviruses remains unconfirmed to date. Nevertheless, HPgV-1 is a bloodborne virus transmitted through percutaneous injuries, blood infusion, sexual contact, and from mother to child (21). The positive results in all blood samples in the beginning of our studies imply a potential for both horizontal and vertical transmission. Previous investigations on GPgV indicated high viral loads in the intestine, although they were not detected in fecal samples (14). However, this information does not rule out the possibility of

horizontal transmission through virus-contaminated feed and water (14). In our research, tracheal swabs only yielded positive results at 21 dpi in two partridges, while cloacal swabs showed positivity at 17 and 21 dpi for one partridge. These findings underline the significance of further investigation due to the existing risk of horizontal transmission. Moreover, research involving Bagaza virus in partridges revealed lower viral loads in blood, feathers, and swabs for grey partridges compared to red-legged partridges. The sustained high viral RNA load in feathers for both species suggests the need for additional investigation in feather sampling (26). The exploration of the virus zoonotic potential and ecological consequences remain incomplete, yet it assumes great importance due to the potential of wild birds to function as reservoirs for numerous diseases (9).

In conclusion, in our study, we inoculated grey partridges with a previously identified novel pegivirus from several encephalitis outbreaks in red-legged partridges in France. The inoculated birds showed mild neurological signs including transient reduced flying behavior. Histological investigations revealed congestions, primarily in spleen, liver, and kidney. Despite the absence of lesions in brain tissue, real-time RT-PCR analysis detected viral RNA, predominantly in brain, spleen, and blood. Additionally, ISH supports the presence of the virus not only in brain, but also in lymphocytes within the liver and spleen. This confirms the lymphotropic nature of pegiviruses, but also a neural tropism. Thus, the present investigation provides further understanding of the pathogenicity and epidemiology of ParPgV in grey partridges. The cause of viral encephalitis outbreaks in red-legged partridges in the field might be linked to ParPgV, as mild neurological signs and a high viral load in brain tissue was shown; however, this cannot be confirmed yet. Hence, it highlights the need of further research on pegivirus in red-legged partridges, given its significant implications for zoonotic potential and ecological consequences in wild birds.

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6. List of figures and tables

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